AOAC INTERNATIONAL

Official Methods Board

Methods to be Reviewed by Official Methods Board (2016)

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2016 AOAC OFFICIAL METHODS BOARD AWARDS

2015 CANDIDATES TO BE REVIEWED FOR

2016 METHOD OF THE YEAR

OFFICIAL METHODS OF ANALYSIS OF AOAC INTERNATIONAL

METHOD OF THE YEAR
OMB may select more than one method in this category each year.

Selection Criteria
The minimum criteria for selection are:

a. The method must have been approved for first or final action within the last three years.
b. Generally, some unique or particularly noteworthy aspect of the method is highlighted as making it worthy of the award, such as innovative technology or application, breadth of applicability, critical need, difficult analysis, and/or range of collaborators.
c. The method demonstrates significant merit in scope or is an innovative approach to an analytical problem.

Selection Process:

a. AOAC staff lists all eligible methods for consideration and forwards that list with supporting documentation (e.g. ERP chair recommendation(s)) to the Chair of the Official Methods Board (OMB).
b. The Chair forwards the list along with any supporting information to the members of the OMB.
c. The OMB selects the Method of the Year. The winner is selected by 2/3 vote. If necessary, the OMB chair may cast tie-breaking vote.

Award
An appropriate letter of appreciation and thanks will be sent to the author(s) of the winning method. The corresponding author will be announced at the appropriate session of the AOAC INTERNATIONAL annual meeting, with presentation of an award. All authors will be acknowledged at the annual meeting, will receive an award and a letter of appreciation. The name of the winner(s), with supporting story, will be carried in the announcement in the *ILM*.

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REVIEWED AND ADOPTED FIRST ACTION METHODS IN 2015

REVIEWED AND APPROVED AS FINAL ACTION METHODS IN 2015

AOAC Official Method 2015.01
Heavy Metals in Food
Inductively Coupled Plasma–Mass Spectrometry
First Action 2015

Note: The following is not intended to be used as a comprehensive training manual. Analytical procedures are written based on the assumption that they will be performed by technicians who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

{Applicable for the determination of heavy metals [arsenic (As), CAS No. 7440-38-2; cadmium (Cd), CAS No. 7440-43-9; lead (Pb), CAS No. 7439-92-1; and mercury (Hg), CAS No. 7439-97-6] at trace levels in food and beverage samples, including solid chocolate, fruit juice, fish, infant formula, and rice, using microwave digestion and inductively coupled plasma–mass spectrometry (ICP-MS).}

Caution: Nitric acid and hydrochloric acid are corrosive. When working with these acids, wear adequate protective gear, including eye protection, gloves with the appropriate resistance, and a laboratory coat. Use an adequate fume hood for all acids.

Hydrogen peroxide is a strong oxidizer and can react violently with organic material to give off oxygen gas and heat. Adequate protective gear should be worn.

Many of the chemicals have toxicities that are not well established and must be handled with care. For all known chemicals used, consult the Material Safety Data Sheet (MSDS) in advance.

The inductively coupled plasma–mass spectrometer emits UV light when the plasma is on. UV resistant goggles should be worn if working near the plasma.

The instrument generates high levels of radio frequency (RF) energy and is very hot when the plasma is on. In the case of an instrument failure, be aware of these potential dangers.

Safely store interference reduction technology (IRT) gases, such as oxygen, in a closed, ventilated cabinet. Use adequate caution with pressurized gases. Prior training or experience is necessary to change any gas cylinders. Oxygen gas can cause many materials to ignite easily.

Following microwave digestion, samples are hot to the touch. Allow the samples to cool to room temperature before opening the digestion vessels to avoid unexpected depressurization and potential release of toxic fumes.

A. Principle

Food samples are thoroughly homogenized and then prepared by microwave digestion and the addition of dilute solutions of gold (Au) and lutetium (Lu). The Au is used to stabilize the Hg in the preparation, and the Lu is used to assess the potential loss of analyte during the microwave digestion process.

A prepared, diluted, aqueous sample digestate is pumped through a nebulizer, where the liquid forms an aerosol as it enters a spray chamber. The aerosol separates into a fine aerosol mist and larger aerosol droplets. The larger droplets exit the spray chamber while the fine mist is transported into the ICP torch.

Inside the ICP torch, the aerosol mist is transported into a high-temperature plasma, where it becomes atomized and ionized as it passes through an RF load coil. The ion stream is then focused by a single ion lens through a cylinder with a carefully controlled electrical field. For instruments equipped with dynamic reaction cell (DRC) or collision cell IRT, the focused ion stream is directed into the reaction/collision cell where, when operating with a pressurized cell, the ion beam will undergo chemical modifications and/or collisions to reduce elemental interferences. When not operating with a pressurized cell, the ion stream will remain focused as it passes through the cell with no chemical modification taking place.

The ion stream is then transported to the quadrupole mass filter, where only ions having a desired mass-to-charge ratio (m/z) are passed through at any moment in time. The ions exiting the mass filter are detected by a solid-state detector and the signal is processed by the data handling system.

B. Equipment

Perform routine preventative maintenance for the equipment used in this procedure.

An ultra-clean laboratory environment is critical for the successful production of quality data at ultra-low levels. All sample preparation must take place in a clean hood (Class 100). Metallic materials should be kept to a minimum in the laboratory and coated with an acrylic polymer gel where possible. Adhesive floor mats should be used at entrances to the laboratory and changed regularly to prevent the introduction of dust and dirt from the outside environment. Wear clean-room gloves and change whenever contact is made with anything non-ultra-clean. The laboratory floor should be wiped regularly to remove any particles without stirring up dust. *Note:* "Ultra-clean" (tested to be low in the analytes of interest) reagents, laboratory supplies, facilities, and sample handling techniques are required to minimize contamination in order to achieve the trace-level detection limits described herein.

(**a**) *Instrumentation*.—ICP-MS instrument, equipped with IRT with a free-running 40 MHz RF generator; and controllers for nebulizer, plasma, auxiliary, and reaction/collision flow control. The quadrupole mass spectrometer has a mass range of 5 to 270 atomic mass units (amu). The turbo molecular vacuum system achieves 10^{-6} torr or better. Recommended ICP-MS components include an RF coil, platinum skimmer and sampler cones, Peltier-cooled quartz cyclonic spray chamber, quartz or sapphire injector, micronebulizer, variable speed peristaltic pump, and various types of tubing (for gases, waste, and peristaltic pump). *Note*: The procedure is written specifically for use with a PerkinElmer ELAN DRC II ICP-MS (www.perkinelmer.com). Equivalent procedures may be performed on any type of ICP-MS instrument with equivalent IRT if the analyst is fully trained in the interpretation of spectral and matrix interferences and procedures for their correction, including the optimization of IRT. For example, collision cell IRT can be used for arsenic determination using helium gas.

(**b**) *Gases*.—High-purity grade liquid argon (>99.996%). Additional gases are required for IRT (such as ultra-x grade, 99.9999% minimum purity oxygen, used for determination of As in DRC mode with some PerkinElmer ICP-MS instruments).

(**c**) *Analytical balance*.—Standard laboratory balance suitable for sample preparation and capable of measuring to 0.1 mg.

(**d**) *Clean-room gloves*.—Tested and certified to be low in the metals of interest.

(**e**) *Microwave digestion system*.—Laboratory microwave digestion system with temperature control and an adequate supply of chemically inert digestion vessels. The microwave should be appropriately vented and corrosion resistant.

(*1*) The microwave digestion system must sense the temperature to within ±2.5°C and automatically adjust the microwave field output power within 2 s of sensing. Temperature sensors should be accurate to ±2°C (including the final reaction temperature of 190°C). Temperature feedback control provides the primary control performance mechanism for the method.

(*2*) The use of microwave equipment with temperature feedback control is required to control the unfamiliar reactions of unique or untested food or beverage samples. These tests may require additional vessel requirements, such as increased pressure capabilities.

(**f**) *Autosampler cups*.—15 and 50 mL; vials are precleaned by soaking in 2–5% (v/v) HNO_3 overnight, rinsed three times with reagent water/deionized water (DIW), and dried in a laminar flow clean hood. For the 50 mL vials, as these are used to prepare standards and bring sample preparations to final volume, the bias and precision of the vials must be assessed and documented prior to use. The recommended procedure for this is as follows:

(*1*) For every case of vials from the same lot, remove 10 vials.

(*2*) Tare each vial on an analytical balance, and then add reagent water up to the 20 mL mark. Repeat procedure by adding reagent water up to the 50 mL mark.

(*3*) Measure and record the mass of reagent water added, and then calculate the mean and RSD of the 10 replicates at each volume.

(*4*) To evaluate bias, the mean of the measurements must be with ±3% of the nominal volume. To evaluate precision, the RSD of the measurements must be ≤3% using the stated value (20 or 50 mL) in place of the mean.

(**g**) *Spatulas*.—To weigh out samples; should be acid-cleaned plastic (ideally Teflon) and cleaned by soaking in 2% (v/v) HNO_3 prior to use.

C. Reagents and Standards

Reagents may contain elemental impurities that could negatively affect data quality. High-purity reagents should always be used. Each reagent lot should be tested and certified to be low in the elements of interest before use.

(**a**) *DIW*.—ASTM Type I; demonstrated to be free from the metals of interest and potentially interfering substances.

(**b**) *Nitric acid (HNO_3)*.—Concentrated; tested and certified to be low in the metals of interest.

(**c**) *Hydrogen peroxide (H_2O_2)*.—Optima grade or equivalent, 30–32% assay.

(**d**) *Stock standard solutions*.—Obtained from a reputable and professional commercial source.

(*1*) *Single-element standards*.—Obtained for each determined metal, as well as for any metals used as internal standards and interference checks.

(*2*) *Second source standard*.—Independent from the single-element standard; obtained for each determined metal.

(*3*) *Multi-element stock standard solution*.—Elements must be compatible and stable in solutions together. Stability is determined by the vendor; concentrations are then verified before use of the standard.

(**e**) *Internal standard solution*.—For analysis of As, Cd, Pb, and Hg in food matrices, an internal standard solution of 40 µg/L rhodium (Rh), indium (In), and thulium (Tm) is recommended. Rh is analyzed in DRC mode for correction of the As signal. In addition, the presence of high levels of elements, such as carbon and chlorine, in samples can increase the effective ionization of the plasma and cause a higher response factor for arsenic in specific samples. This potential interference is addressed by the on-line addition of acetic acid (or another carbon source, such as methanol), which greatly increases the effective ionization of incompletely ionized analytes, and decreases the potential increase caused by sample characteristics. The internal standard solution should be prepared in 20% acetic acid.

Table 2015.01A. Recommended concentrations for the calibration curve

Standard	As, µg/L	Cd, µg/L	Pb, µg/L	Hg, µg/L
0	0.00	0.00	0.000	0.00
1	0.01	0.01	0.005	0.01
2	0.02	0.02	0.010	0.05
3	0.10	0.10	0.050	0.10
4	0.50	0.50	0.250	0.50
5	5.00	5.00	2.500	2.00
6	20.00	20.00	10.000	5.00

(**f**) *Calibration standards*.—Fresh calibration standards should be prepared every day, or as needed.

(*1*) Dilute the multi-element stock standard solutions into 50 mL precleaned autosampler vials with 5% HNO_3 in such a manner as to create a calibration curve. The lowest calibration standard (STD 1) should be equal to or less than the limit of quantitation (LOQ) when recalculated in units specific to the reported sample results.

(*2*) *See* Table **2015.01A** for recommended concentrations for the calibration curve.

(**g**) *Initial calibration verification (ICV) solution*.—Made up from second source standards in order to verify the validity of the calibration curve.

(**h**) *Calibration solutions*.—Daily optimization, tuning, and dual detector calibration solutions, as needed, should be prepared and analyzed per the instrument manufacturer's suggestions.

(**i**) *Certified Reference Materials (CRMs)*.—CRMs should preferably match the food matrix type being analyzed and contain the elements of interest at certified concentrations above the LOQ. Recommended reference materials include NIST SRM 1568a (Rice Flour), NIST SRM 1548a (Typical Diet), NRCC CRM DORM-3 (Dogfish Muscle), and NIST SRM 2976 (Mussel Tissue).

(**j**) *Spiking solution*.—50 mg/L Au and Lu in 5% (v/v) HNO_3. Prepared from single-element standards.

D. Contamination and Interferences

(**a**) Well-homogenized samples and small reproducible aliquots help minimize interferences.

(**b**) *Contamination*.—(*1*) Contamination of the samples during sample handling is a great risk. Extreme care should be taken to avoid this. Potential sources of contamination during sample handling include using metallic or metal-containing homogenization equipment, laboratory ware, containers, and sampling equipment.

(*2*) Contamination of samples by airborne particulate matter is a concern. Sample containers must remain closed as much as possible. Container lids should only be removed briefly and in a

clean environment during sample preservation and processing, so that exposure to an uncontrolled environment is minimized.

(**c**) *Laboratory.*—(*1*) All laboratory ware (including pipet tips, ICP-MS autosampler vials, sample containers, extraction apparatus, and reagent bottles) should be tested for the presence of the metals of interest. If necessary, the laboratory ware should be acid-cleaned, rinsed with DIW, and dried in a Class 100 laminar flow clean hood.

(*2*) All autosampler vials should be cleaned by storing them in 2% (v/v) HNO_3 overnight and then rinsed three times with DIW. Then dry vials in a clean hood before use. Glass volumetric flasks should be soaked in about 5% HNO_3 overnight prior to use.

(*3*) All reagents used for analysis and sample preparation should be tested for the presence of the metals of interest prior to use in the laboratory. Due to the ultra-low detection limits of the method, it is imperative that all the reagents and gases be as low as possible in the metals of interest. It is often required to test several different sources of reagents until an acceptable source has been found. Metals contamination can vary greatly from lot to lot, even when ordering from the same manufacturer.

(*4*) Keep the facility free from all sources of contamination for the metals of interest. Replace laminar flow clean hood HEPA filters with new filters on a regular basis, typically once a year, to reduce airborne contaminants. Metal corrosion of any part of the facility should be addressed and replaced. Every piece of apparatus that is directly or indirectly used in the processing of samples should be free from contamination for the metals of interest.

(**d**) *Elemental interferences*.—Interference sources that may inhibit the accurate collection of ICP-MS data for trace elements are addressed below.

(*1*) *Isobaric elemental interferences*.—Isotopes of different elements that form singly or doubly charged ions of the same *m/z* and cannot be resolved by the mass spectrometer. Data obtained with isobaric overlap must be corrected for that interference.

(*2*) *Abundance sensitivity*.—Occurs when part of an elemental peak overlaps an adjacent peak. This often occurs when measuring a small *m/z* peak next to a large *m/z* peak. The abundance sensitivity is affected by ion energy and quadrupole operating pressure. Proper optimization of the resolution during tuning will minimize the potential for abundance sensitivity interferences.

(*3*) *Isobaric polyatomic interferences.*—Caused by ions, composed of multiple atoms, which have the same *m/z* as the isotope of interest, and which cannot be resolved by the mass spectrometer. These ions are commonly formed in the plasma or the interface system from the support gases or sample components. The objective of IRT is to remove these interferences, making the use of correction factors unnecessary when analyzing an element in DRC mode. Elements not determined in DRC mode can be corrected by using correction equations in the ICP-MS software.

(**e**) *Physical interferences.*—(*1*) Physical interferences occur when there are differences in the response of the instrument from the calibration standards and the samples. Physical interferences are associated with the physical processes that govern the transport of sample into the plasma, sample conversion processes in the plasma, and the transmission of ions through the plasma-mass spectrometer interface.

(*2*) Physical interferences can be associated with the transfer of solution to the nebulizer at the point of nebulization, transport of aerosol to the plasma, or during excitation and ionization processes in the plasma. High levels of dissolved solids in a sample can result in physical interferences. Proper internal standardization (choosing internal standards that have analytical behavior similar to the associating elements) can compensate for many physical interferences.

Table 2015.01B. Recommended isotopes for analysis

Element	Isotope, amu	Isotopic abundance, %	Potential interferences
Cd	111	13	MoO^+
	114	29	MoO^+, Sn^+
Hg	200	23	WO^+
	202	30	WO^+
Pb[a]	Sum of 206, 207, and 208	99	OsO^+

[a] Allowance for isotopic variability of lead isotopes.

(**f**) *Resolution of interferences.*—(*1*) For elements that are subject to isobaric or polyatomic interferences (such as As), it is advantageous to use the DRC mode of the instrument. This section specifically describes a method of using IRT for interference removal for As using a PerkinElmer DRC II and oxygen as the reaction gas. Other forms of IRT may also be appropriate.

(*a*) Arsenic, which is monoisotopic, has an *m/z* of 75 and is prone to interferences from many sources, most notably from chloride (Cl), which is common in many foods (e.g., salt). Argon (Ar), used in the ICP-MS plasma, forms a polyatomic interference with Cl at *m/z* 75 [$^{35}Cl + {}^{40}Ar = {}^{75}(ArCl)$].

(*b*) When arsenic reacts with the oxygen in the DRC cell, $^{75}As^{16}O$ is formed and measured at *m/z* 91, which is free of most interferences. The potential ^{91}Zr interference is monitored for in the following ways: ^{90}Zr and ^{94}Zr are monitored for in each analytical run, and if a significant Zr presence is detected, then $^{75}As^{16}O$ measured at *m/z* 91 is evaluated against the ^{75}As result. If a significant discrepancy is present, then samples may require analysis using alternative IRT, such as collision cell technology (helium mode).

(*c*) Instrument settings used (for PerkinElmer DRC II): DRC settings for $^{91}(AsO)$ and ^{103}Rh include an RPq value of 0.7 and a cell gas flow rate of 0.6 L/min. Cell conditions, especially cell gas flow rates, may be optimized for specific analyte/matrix combinations, as needed. In such cases, the optimized methods will often have slightly different RPq and cell gas flow values.

(*2*) For multi-isotopic elements, more than one isotope should be measured to monitor for potential interferences. For reporting purposes, the most appropriate isotope should be selected based on review of data for matrix interferences and based on the sensitivity (or relative abundance) of each isotope. The table below lists the recommended isotopes to measure. Low abundance isotopes are not recommended for this method as it is specifically applicable for ultra-low level concentrations (8–10 ppb LOQs). *See* Table **2015.01B**.

(**g**) *Memory effects*.—Minimize carryover of elements in a previous sample in the sample tubing, cones, torch, spray chamber, connections, and autosampler probe by rinsing the instrument with a reagent blank after samples high in metals concentrations are analyzed. Memory effects for Hg can be minimized through the addition of Au to all standard, samples, and quality control (QC) samples.

Table 2015.01C. Digestion program for Berghof Speedwave 4 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	145	1	1
2	50	1	1
3	145	1	1
4	170	1	10
5	190	1	10

E. Sample Handling and Storage

(**a**) Food and beverage samples should be stored in their typical commercial storage conditions (either frozen, refrigerated, or at room temperature) until analysis. Samples should be analyzed within 6 months of preparation.

(**b**) If food or beverage samples are subsampled from their original storage containers, ensure that containers are free from contamination for the elements of concern.

F. Sample Preparation

(**a**) Weigh out sample aliquots (typically 0.25 g of as-received or wet sample) into microwave digestion vessels.

(**b**) Add 4 mL of concentrated HNO_3 and 1 mL of 30% hydrogen peroxide (H_2O_2) to each digestion vessel.

(**c**) Add 0.1 mL of the 50 mg/L Au + Lu solution to each digestion vessel.

(**d**) Cap the vessels securely (and insert into pressure jackets, if applicable). Place the vessels into the microwave system according to the manufacturer's instructions, and connect the appropriate temperature and/or pressure sensors.

(**e**) Samples are digested at a minimum temperature of 190°C for a minimum time of 10 min. Appropriate ramp times and cool down times should be included in the microwave program, depending on the sample type and model of microwave digestion system. Microwave digestion is achieved using temperature feedback control. Microwave digestion programs will vary depending on the type of microwave digestion system used. When using this mechanism for achieving performance-based digestion targets, the number of samples that may be simultaneously digested may vary. The number will depend on the power of the unit, the number of vessels, and the heat loss characteristics of the vessels. It is essential to ensure that all vessels reach at least 190°C and be held at this temperature for at least 10 min. The monitoring of one vessel as a control for the batch/carousel may not accurately reflect the temperature in the other vessels, especially if the samples vary in composition and/or sample mass. Temperature measurement and control will depend on the particular microwave digestion system.

(*1*) *Note*: a predigestion scheme for samples that react vigorously to the addition of the acid may be required.

(*2*) The method performance data presented in this method was produced using a Berghof Speedwave 4 microwave digestion system, with the program listed in Table **2015.01C** (steps 1 and 2 are a predigestion step).

Table 2015.01D. Digestion program for CEM MARS 6 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	190	20	10
2	Cool down	NA	10

(*3*) Equivalent results were achieved using the program listed in Table **2015.01D** on a CEM MARS 6 microwave digestion system using the 40-position carousel and 55 mL Xpress digestion vessels.

(*4*) For infant formula samples, the program described in Table **2015.01E** has been shown to work effectively.

Table 2015.01E. Digestion program for infant formula

Step	Temp., °C	Ramp, min	Hold, min
1	180	20	20
2	Cool down	NA	20
3	200	20	20
4	Cool down	NA	20

(**f**) Allow vessels to cool to room temperature and slowly open. Open the vessels carefully, as residual pressure may remain and digestate spray is possible. Pour the contents of each vessel into an acid-cleaned 50 mL HDPE centrifuge tube and dilute with DIW to a final volume of 20 mL.

(**g**) Digestates are diluted at least 4x prior to analysis with the 1% (v/v) HNO_3 diluent. When the metals concentration of a sample is unknown, the samples may be further diluted or analyzed using a total quantification method prior to being analyzed with a comprehensive quantitative method. This protects the instrument and the sample introduction system from potential contamination and damage.

(**h**) Food samples high in calcium carbonate ($CaCO_3$) will not fully digest. In such cases, the CRM can be used as a gauge for an appropriate digestion time.

(**i**) QC samples to be prepared with the batch (a group of samples and QC samples that are prepared together) include a minimum of three method blanks, duplicate for every 10 samples, matrix spike/matrix spike duplicate (MS/MSD) for every 10 samples, blank spike, and any matrix-relevant CRMs that are available.

G. Procedure

(**a**) *Instrument startup*.—(*1*) Instrument startup routine and initial checks should be performed per manufacturer recommendations.

(*2*) Ignite the plasma and start the peristaltic pump. Allow plasma and system to stabilize for at least 30 min.

(**b**) *Optimizations*.—(*1*) Perform an optimization of the sample introduction system (e.g., *X*-*Y* and *Z* optimizations) to ensure maximum sensitivity.

(*2*) Perform an instrument tuning or mass calibration routine whenever there is a need to modify the resolution for elements, or monthly (at a minimum), to ensure the instrument's quadrupole mass filtering performance is adequate. Measured masses should be ±0.1 amu of the actual mass value, and the resolution (measured peak width) should conform to manufacturer specifications.

(*3*) Optimize the nebulizer gas flow for best sensitivity while maintaining acceptable oxide and double-charged element formation ratios.

(*4*) Perform a daily check for instrument sensitivity, oxide formation ratios, double-charged element formation ratios, and background. If the performance check is not satisfactory, additional optimizations (a "full optimization") may be necessary.

Table 2015.01F. Summary of quality control samples

QC sample	Measure	Minimum frequency	Acceptance criteria	Corrective action
Calibration standards	Linearity of the calibration curve	Analyzed once per analytical day	Correlation coefficient ≥0.995, 1st standard ≤MRL, low standard recovery = 75–125%, all other standard recoveries = 80–120%	Reanalyze suspect calibration standard. If criteria still not met, then re-prepare standards and recalibrate the instrument.
Internal standards	Variation in sample properties between samples and standards	Each standard, blank, and sample is spiked with internal standard	60–125% recovery compared to calibration blank	If the responses of the internal standards in the following CCB are within the limit, rerun the sample at an additional 2x dilution. If not, then samples must be reanalyzed with a new calibration.
Lu digestion check spike	Assessment of potential loss during digestion	Added to every digested samples	Recovery ≥75%	Re-prepare the sample
Initial calibration verification (ICV)	Independent check of system performance	One following instrument calibration	Recovery = 90–110%	Correct problem prior to continuing analysis. Recalibrate if necessary.
Continuing calibration verification (CCV)	Accuracy	At beginning and end of analysis and one per 10 injections	Recovery = 85–115%	Halt analysis, correct problem, recalibrate, and reanalyze affected samples
Method blanks (MB)	Contamination from reagents, lab ware, etc.	Minimum of three per batch	Mean ≤ MRL; SD ≤ MDL or MBs <1/10th sample result	Determine and eliminate cause of contamination. Affected samples must be re-prepared and reanalyzed.
Method duplicates (MD)	Method precision within a given matrix	Minimum of one per 10 samples	RPD ≤ 30% or ±2x LOQ if results ≤5x LOQ	If RPD criteria not met, then sample may be re-prepared and reanalyzed, but this is not required. Sample matrix may be inhomogeneous. A post-digestion duplicate (PDD) can be analyzed to evaluate instrument precision.
Matrix spikes/matrix spike duplicates (MS/MSD)	Method accuracy and precision within a given matrix	Minimum of one per 10 samples	Recovery = 70–130% and RPD ≤ 30%	If RPD >30%, results must be qualified
Post-preparation spike (PS)	Check for matrix interference	When required (samples spiked too low/high, dilution test fails, etc.)	Recovery = 75–125%	Analyze samples using MSA or results flagged accordingly
Laboratory fortified blank (LFB) or blank spike (BS)	Method accuracy	Minimum of one per batch	Recovery = 75–125%	If LFB recovery is outside of the control limit, then batch must be re-prepared and reanalyzed
Certified Reference Material (CRM)	Method accuracy	Must be matrix-matched to samples; minimum of one per batch	Recovery = 75–125% unless limits set by CRM manufacturer are greater or element/CRM specific limits have been established	If CRM true value is ≥5x the LOQ and recovery is outside of the control limit, then batch must be re-prepared and reanalyzed

(**c**) *Internal standardization and calibration*.—(*1*) Following precalibration optimizations, prepare and analyze the calibration standards prepared as described in **C**(**e**).

(*2*) Use internal standardization in all analyses to correct for instrument drift and physical interferences. Refer to **D**(**e**)(*2*). Internal standards must be present in all samples, standards, and blanks at identical concentrations. Internal standards can be added using a second channel of the peristaltic pump to produce a responses that is clear of the pulse-to-analog detector interface.

(*3*) Multiple isotopes for some analytes may be measured, with only the most appropriate isotope (as determined by the analyst) being reported.

(*4*) Use IRT for the quantification of As using the Rh internal standard.

(**d**) *Sample analysis*.—(*1*) Create a method file for the ICP-MS.

(*2*) Enter sample and calibration curve information into the ICP-MS software.

(*3*) Calibrate the instrument and ensure the resulting standard recoveries and correlation coefficients meet specifications (**H**).

(*4*) Start the analysis of the samples.

(*5*) Immediately following the calibration, an initial calibration blank (ICB) should be analyzed. This demonstrates that there is no carryover of the analytes of interest and that the analytical system is free from contamination.

(*6*) Immediately following the ICB, an ICV should be analyzed. This standard must be prepared from a different source than the calibration standards.

(*7*) A minimum of three reagent/instrument blanks should be analyzed following the ICV. These instrument blanks can be used to assess the background and variability of the system.

(*8*) A continuing calibration verification (CCV) standard should be analyzed after every 10 injections and at the end of the run. The CCV standard should be a mid-range calibration standard.

(*9*) An instrument blank should be analyzed after each CCV (called a continuing calibration blank, or CCB) to demonstrate that there is no carryover and that the analytical system is free from contamination.

(*10*) Method of Standard Additions (MSA) calibration curves may be used any time matrix interferences are suspected.

(*11*) Post-preparation spikes (PS) should be prepared and analyzed whenever there is an issue with the MS recoveries.

(**e**) Export and process instrument data.

H. Quality Control

(**a**) The correlation coefficients of the weighted-linear calibration curves for each element must be ≥0.995 to proceed with sample analysis.

(**b**) The percent recovery of the ICV standard should be 90–110% for each element being determined.

(**c**) Perform instrument rinses after any samples suspected to be high in metals, and before any method blanks, to ensure baseline sensitivity has been achieved. Run these rinses between all samples in the batch to ensure a consistent sampling method.

(**d**) Each analytical or digestion batch must have at least three preparation (or method) blanks associated with it if method blank correction is to be performed. The blanks are treated the same as the samples and must go through all of the preparative steps. If method blank correction is being used, all of the samples in the batch should be corrected using the mean concentration of these blanks. The estimated method detection limit (EMDL) for the batch is equal to 3 times the standard deviation (SD) of these blanks.

(**e**) For every 10 samples (not including quality control samples), a matrix duplicate (MD) sample should be analyzed. This is a duplicate of a sample that is subject to all of the same preparation and analysis steps as the original sample. Generally, the relative percent difference (RPD) for the replicate should be ≤30% for all food samples if the sample concentrations are greater than 5 times the LOQ. RPD is calculated as shown below. An MSD may be substituted for the MD, with the same control limits.

$$RPD = 200 \times \frac{|S1 - S2|}{S1 + S2}$$

where S1 = concentration in the first sample and S2 = concentration in the duplicate.

(**f**) For every 10 samples (not including quality control samples), an MS and MSD should be performed. The percent recovery of the spikes should be 70–130% with an RPD ≤30% for all food samples.

(*1*) If the spike recovery is outside of the control limits, an MSA curve that has been prepared and analyzed may be used to correct for the matrix effect. Samples may be corrected by the slope of the MSA curve if the correlation coefficient of the MSA curve is ≥0.995.

(*a*) The MSA technique involves adding known amounts of standard to one or more aliquots of the processed sample solution. This technique attempts to compensate for a sample constituent that enhances or depresses the analyte signal, thus producing a different slope from that of the calibration standards. It will not correct for additive interferences which cause a baseline shift.

(*b*) The best MSA results can be obtained by using a series of standard additions. To equal volumes of the sample are added a series of standard solutions containing different known quantities of the analyte(s), and all solutions are diluted to the same final volume. For example, addition 1 should be prepared so that the resulting concentration is approximately 50% of the expected concentration of the native sample. Additions 2 and 3 should be prepared so that the concentrations are approximately 100% and 150%, respectively, of the expected native sample concentration. Determine the concentration of each solution and then plot on the vertical axis of a graph, with the concentrations of the known standards plotted on the horizontal axis. When the resulting line is extrapolated to zero absorbance, the point of interception of the abscissa is calculated MSA-corrected concentration of the analyte in the sample. A linear regression program may be used to obtain the intercept concentration.

(*c*) For results of the MSA technique to be valid, take into consideration the following limitations:

(*i*) The apparent concentrations from the calibration curve must be linear (0.995 or greater) over the concentration range of concern.

(*ii*) The effect of the interference should not vary as the ratio of analyte concentration to sample matrix changes, and the MSA curve should respond in a similar manner as the analyte.

(*2*) If the sample concentration levels are sufficiently high, the sample may be diluted to reduce the matrix effect. Samples should be diluted with the 1% (v/v) HNO_3 diluent. For example, to dilute a sample by a 10x dilution factor, pipette 1 mL of the digested sample into an autosampler vial, and add 9 mL of the 1% (v/v) HNO_3 diluent. MS/MSD sets should be performed at the same dilution factor as the native sample.

(*3*) Spike at 1–10 times the level of a historical sample of the same matrix type, or, if unknown, spike at 1–5 times a typical value for the matrix. Spiking levels should be no lower than 10 times the LOQ.

Table 2015.01G. Method blank results and LOD/LOQ, µg/kg

Method blanks	^{91}(AsO)	^{111}Cd	^{114}Cd	Pb	^{200}Hg	^{202}Hg
MB-01	2.83	0.229	0.270	1.90	1.61	0.95
MB-02	1.48	–0.088	0.270	0.14	1.48	1.13
MB-03	1.80	0.007	0.115	0.13	0.76	0.25
MB-04	1.03	0.154	0.288	0.12	1.46	0.33
MB-05	1.43	0.010	0.259	1.84	1.28	0.27
MB-06	1.07	0.105	0.096	3.02	0.87	0.76
MB-07	2.31	–0.002	0.297	2.67	0.89	0.44
MB-08	1.20	0.285	0.200	4.24	0.55	0.28
MB-09	1.05	0.002	0.182	0.09	0.96	0.25
MB-10	2.12	0.047	0.150	0.19	0.71	0.02
MB-11	2.09	–0.145	0.226	0.12	0.64	0.57
MB-12	1.44	0.037	0.165	0.18	0.45	0.50
MB-13	0.70	–0.122	0.160	0.17	0.81	0.19
MB-14	1.12	–0.001	0.074	0.14	0.85	0.21
MB-15	2.33	0.097	0.207	0.11	0.18	0.17
MB-16	1.53	–0.117	0.146	0.16	1.33	1.09
MB-17	1.79	–0.070	0.180	0.03	3.46	2.19
MB-18	1.90	0.049	0.115	0.06	3.30	2.36
MB-19	1.18	0.043	0.224	0.39	4.01	2.78
MB-20	1.24	–0.060	0.199	0.07	0.99	0.56
MB-21	0.92	0.165	0.120	0.03	0.73	0.33
MB-22	1.69	0.005	0.186	0.09	0.60	0.25
MB-23	2.13	0.171	0.152	0.08	0.41	–0.23
SD	0.54	0.113	0.063	1.18	1.01	0.77
LOD	1.6	0.50[a]	0.50[a]	3.5	3.0	2.3
LOQ	3.3	1.60[a]	1.60[a]	7.1	6.0	4.6

[a] Adjusted to conform to lowest calibration point.

(**g**) Percent recoveries of the CRMs should be 75–125% of their certified value.

(**h**) Percent recoveries of the CCV standards should be within 85–115%. Sample results may be CCV-corrected using the mean recovery of the bracketing CCVs. This should only be done after careful evaluation of the data. The instrument should show a trending drift of CCV recoveries and not just a few anomalous outliers.

(**i**) CCBs should be monitored for the effects of carryover and for possible system contamination. If carryover of the analyte at levels greater than 10 times the MDL is observed, the sample results may not be reportable.

(**j**) Absolute response of any one internal standard should not vary from the original response in the calibration blank by more than 60–125%. Some analytical samples, such as those containing concentrations of the internal standard and tissue digestates, can have a serious effect on the internal standard intensities, but this does not necessarily mean that the analytical system is out of control. In some situations, it is appropriate to reprocess the samples using a different internal standard monitored in the analysis. The data should be carefully evaluated before doing this.

Table 2015.01H. Sample-specific LOQs

Sample	LOQ, µg/kg (as received)			
	As	Cd	Pb	Hg
Infant formula	2	1	4	3
Chocolate	4	2	8	6
Rice flour	4	2	8	6
Fruit juice	1	1	2	2

(**k**) The recovery of the Lu that was spiked into the sample preparation prior to digestion should be evaluated to assess any potential loss of analyte during the process. The concentration of Lu in the sample preparation is 0.25 mg/L, and for samples diluted 4x at the instrument, this is equivalent to 62.5 µg/L at the instrument (if samples are diluted more than 4x, this must be taken into account). The Lu recovery should be no less than 75% of the original spiked concentration.

(**l**) Refer to Table **2015.01F** for a summary of all recommended quality control samples, minimum frequency at which they are to be analyzed, acceptance criteria for each, and appropriate corrective action if the acceptance criteria are not met.

I. Method Performance

(**a**) Limit of detection (LOD) and LOQ were determined through the analysis of 23 method blanks (*see* Table **2015.01G**). LOD was calculated as 3 times the SD of the results of the blanks, and LOQ was calculated as 2 times the value of the LOD, except where the resulting LOQ would be less than the lowest calibration point, in which case LOQ was elevated and set at the lowest calibration point and LOD was calculated as 1/3 of the LOQ. All LOQs achieved are ≤10 µg/kg for all food matrices and ≤8 µg/kg for liquid matrices, such as infant formula.

(**b**) Sample-specific LOQs for several matrices, based on LOQs determined by the default method, and adjusted for changes in sample mass for particular samples, are shown in Table **2015.01H**. Values have been rounded up to the nearest part-per-billion.

(**c**) Numerous relevant CRMs were analyzed to establish method accuracy. Example percent recoveries are provided in Table **2015.01I** (recoveries have been omitted for CRMs that do not provide a certified value or if the certified value is less than the LOQ).

Table 2015.01I. Recoveries for numerous relevant CRMs

Certified Reference Material	As, %	Cd, %	Pb, %	Hg, %
DOLT-4 Dogfish Liver	104	97	87	114
DORM-3 Fish Protein	105	109	94	114
DORM-4 Fish Protein	105	91	91	81
NIST 1548a Typical Diet	103	95	113	NA
NIST 1568a Rice Flour	98	99	NA	NA
NIST 1946 Lake Superior Fish Tissue	119	NA	NA	101
TORT-2 Lobster Hepatopancreas	109	104	95	116
TORT-3 Lobster Hepatopancreas	113	89	86	86

Table 2015.01J. AOAC SMPR 2012.007 (ref. 1)

Concn range, µg/kg	Repeatability, %	Reproducibility, %	Recovery, %
LOQ–100	15	32	60–115
100–1000	11	16	80–115
>1000	7.3	8	80–115

(**d**) *Standard Method Performance Requirements* (AOAC SMPR® 2012.007; 1) for repeatability, reproducibility, and recovery for the method are shown in the Table **2015.01J**. *See* Appendix A (available on the *J. AOAC Int*. website as supplemental material, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac) for detailed method performance information supporting acceptance of the method.

(**e**) *See* Appendix A for detailed method performance information supporting acceptance of the method. Method validation samples were prepared and analyzed for all applicable matrices. In general, all SMPR criteria were met for As, Cd, Hg, and Pb in the matrices apple juice, infant formula, cocoa powder, and rice flour.

References: (1) AOAC SMPR 2012.007
J. AOAC Int. **96**, 704(2013)
DOI: 10.5740/jaoac.int.2012.007

J. AOAC Int. **98**, 1113(2015)
DOI: 10.5740/jaoac.int.2015.01

Posted: September 9, 2015

OFFICIAL METHODS

Determination of Heavy Metals in Food by Inductively Coupled Plasma–Mass Spectrometry: First Action 2015.01

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Submitted for publication February 2015.
Adopted as a First Action Official Method by the Expert Review Panel on Heavy Metals and approved by the Stakeholder Panel on Strategic Food Analytical Methods(SPSFAM).
Approved on: February 11, 2015.
Appendix A is available on the *J. AOAC Int.* website as supplemental material, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI: 10.5740/jaoac.int.2015.01

Note: The following is not intended to be used as a comprehensive training manual. Analytical procedures are written based on the assumption that they will be performed by technicians who are formally trained in at least the basic principles of chemical analysis and in the use of the subject technology.

{Applicable for the determination of heavy metals [arsenic (As), CAS No. 7440-38-2; cadmium (Cd), CAS No. 7440-43-9; lead (Pb), CAS No. 7439-92-1; and mercury (Hg), CAS No. 7439-97-6] at trace levels in food and beverage samples, including solid chocolate, fruit juice, fish, infant formula, and rice, using microwave digestion and inductively coupled plasma–mass spectrometry (ICP-MS).}

Caution: Nitric acid and hydrochloric acid are corrosive. When working with these acids, wear adequate protective gear, including eye protection, gloves with the appropriate resistance, and a laboratory coat. Use an adequate fume hood for all acids.

Hydrogen peroxide is a strong oxidizer and can react violently with organic material to give off oxygen gas and heat. Adequate protective gear should be worn.

Many of the chemicals have toxicities that are not well established and must be handled with care. For all known chemicals used, consult the Material Safety Data Sheet (MSDS) in advance.

The inductively coupled plasma–mass spectrometer emits UV light when the plasma is on. UV resistant goggles should be worn if working near the plasma.

The instrument generates high levels of radio frequency (RF) energy and is very hot when the plasma is on. In the case of an instrument failure, be aware of these potential dangers.

Safely store interference reduction technology (IRT) gases, such as oxygen, in a closed, ventilated cabinet. Use adequate caution with pressurized gases. Prior training or experience is necessary to change any gas cylinders. Oxygen gas can cause many materials to ignite easily.

Following microwave digestion, samples are hot to the touch. Allow the samples to cool to room temperature before opening the digestion vessels to avoid unexpected depressurization and potential release of toxic fumes.

A. Principle

Food samples are thoroughly homogenized and then prepared by microwave digestion and the addition of dilute solutions of gold (Au) and lutetium (Lu). The Au is used to stabilize the Hg in the preparation, and the Lu is used to assess the potential loss of analyte during the microwave digestion process.

A prepared, diluted, aqueous sample digestate is pumped

through a nebulizer, where the liquid forms an aerosol as it enters a spray chamber. The aerosol separates into a fine aerosol mist and larger aerosol droplets. The larger droplets exit the spray chamber while the fine mist is transported into the ICP torch.

Inside the ICP torch, the aerosol mist is transported into a high-temperature plasma, where it becomes atomized and ionized as it passes through an RF load coil. The ion stream is then focused by a single ion lens through a cylinder with a carefully controlled electrical field. For instruments equipped with dynamic reaction cell (DRC) or collision cell IRT, the focused ion stream is directed into the reaction/collision cell where, when operating with a pressurized cell, the ion beam will undergo chemical modifications and/or collisions to reduce elemental interferences. When not operating with a pressurized cell, the ion stream will remain focused as it passes through the cell with no chemical modification taking place.

The ion stream is then transported to the quadrupole mass filter, where only ions having a desired mass-to-charge ratio (*m/z*) are passed through at any moment in time. The ions exiting the mass filter are detected by a solid-state detector and the signal is processed by the data handling system.

B. Equipment

Perform routine preventative maintenance for the equipment used in this procedure.

An ultra-clean laboratory environment is critical for the successful production of quality data at ultra-low levels. All sample preparation must take place in a clean hood (Class 100). Metallic materials should be kept to a minimum in the laboratory and coated with an acrylic polymer gel where possible. Adhesive floor mats should be used at entrances to the laboratory and changed regularly to prevent the introduction of dust and dirt from the outside environment. Wear clean-room gloves and change whenever contact is made with anything non-ultra-clean. The laboratory floor should be wiped regularly to remove any particles without stirring up dust. *Note:* "Ultra-clean" (tested to be low in the analytes of interest) reagents, laboratory supplies, facilities, and sample handling techniques are required to minimize contamination in order to achieve the trace-level detection limits described herein.

(**a**) *Instrumentation*.—ICP-MS instrument, equipped with IRT with a free-running 40 MHz RF generator; and controllers for nebulizer, plasma, auxiliary, and reaction/collision flow control. The quadrupole mass spectrometer has a mass range of 5 to 270 atomic mass units (amu). The turbo molecular vacuum system achieves 10^{-6} torr or better. Recommended ICP-MS components include an RF coil, platinum skimmer and sampler cones, Peltier-cooled quartz cyclonic spray chamber, quartz or sapphire injector, micronebulizer, variable speed peristaltic pump, and various types of tubing (for gases, waste, and peristaltic pump). *Note*: The procedure is written specifically for use with a PerkinElmer ELAN DRC II ICP-MS (www.perkinelmer.com). Equivalent procedures may be performed on any type of ICP-MS instrument with equivalent IRT if the analyst is fully trained in the interpretation of spectral and matrix interferences and procedures for their correction, including the optimization of IRT. For example, collision cell IRT can be used for arsenic determination using helium gas.

(**b**) *Gases*.—High-purity grade liquid argon (>99.996%). Additional gases are required for IRT (such as ultra-x grade, 99.9999% minimum purity oxygen, used for determination of As in DRC mode with some PerkinElmer ICP-MS instruments).

(**c**) *Analytical balance*.—Standard laboratory balance suitable for sample preparation and capable of measuring to 0.1 mg.

(**d**) *Clean-room gloves*.—Tested and certified to be low in the metals of interest.

(**e**) *Microwave digestion system*.—Laboratory microwave digestion system with temperature control and an adequate supply of chemically inert digestion vessels. The microwave should be appropriately vented and corrosion resistant.

(*1*) The microwave digestion system must sense the temperature to within ±2.5°C and automatically adjust the microwave field output power within 2 s of sensing. Temperature sensors should be accurate to ±2°C (including the final reaction temperature of 190°C). Temperature feedback control provides the primary control performance mechanism for the method.

(*2*) The use of microwave equipment with temperature feedback control is required to control the unfamiliar reactions of unique or untested food or beverage samples. These tests may require additional vessel requirements, such as increased pressure capabilities.

(**f**) *Autosampler cups*.—15 and 50 mL; vials are precleaned by soaking in 2–5% (v/v) HNO_3 overnight, rinsed three times with reagent water/deionized water (DIW), and dried in a laminar flow clean hood. For the 50 mL vials, as these are used to prepare standards and bring sample preparations to final volume, the bias and precision of the vials must be assessed and documented prior to use. The recommended procedure for this is as follows:

(*1*) For every case of vials from the same lot, remove 10 vials.

(*2*) Tare each vial on an analytical balance, and then add reagent water up to the 20 mL mark. Repeat procedure by adding reagent water up to the 50 mL mark.

(*3*) Measure and record the mass of reagent water added, and then calculate the mean and RSD of the 10 replicates at each volume.

(*4*) To evaluate bias, the mean of the measurements must be with ±3% of the nominal volume. To evaluate precision, the RSD of the measurements must be ≤3% using the stated value (20 or 50 mL) in place of the mean.

(**g**) *Spatulas*.—To weigh out samples; should be acid-cleaned plastic (ideally Teflon) and cleaned by soaking in 2% (v/v) HNO_3 prior to use.

C. Reagents and Standards

Reagents may contain elemental impurities that could negatively affect data quality. High-purity reagents should always be used. Each reagent lot should be tested and certified to be low in the elements of interest before use.

(**a**) *DIW*.—ASTM Type I; demonstrated to be free from the metals of interest and potentially interfering substances.

(**b**) *Nitric acid (HNO_3)*.—Concentrated; tested and certified to be low in the metals of interest.

(**c**) *Hydrogen peroxide (H_2O_2)*.—Optima grade or equivalent, 30–32% assay.

(**d**) *Stock standard solutions*.—Obtained from a reputable and professional commercial source.

(*1*) *Single-element standards*.—Obtained for each determined metal, as well as for any metals used as internal standards and interference checks.

Table 2015.01A. Recommended concentrations for the calibration curve

Standard	As, µg/L	Cd, µg/L	Pb, µg/L	Hg, µg/L
0	0.00	0.00	0.000	0.00
1	0.01	0.01	0.005	0.01
2	0.02	0.02	0.010	0.05
3	0.10	0.10	0.050	0.10
4	0.50	0.50	0.250	0.50
5	5.00	5.00	2.500	2.00
6	20.00	20.00	10.000	5.00

(*2*) *Second source standard*.—Independent from the single-element standard; obtained for each determined metal.

(*3*) *Multi-element stock standard solution*.—Elements must be compatible and stable in solutions together. Stability is determined by the vendor; concentrations are then verified before use of the standard.

(**e**) *Internal standard solution*.--For analysis of As, Cd, Pb, and Hg in food matrices, an internal standard solution of 40 µg/L rhodium (Rh), indium (In), and thulium (Tm) is recommended. Rh is analyzed in DRC mode for correction of the As signal. In addition, the presence of high levels of elements, such as carbon and chlorine, in samples can increase the effective ionization of the plasma and cause a higher response factor for arsenic in specific samples. This potential interference is addressed by the on-line addition of acetic acid (or another carbon source, such as methanol), which greatly increases the effective ionization of incompletely ionized analytes, and decreases the potential increase caused by sample characteristics. The internal standard solution should be prepared in 20% acetic acid.

(**f**) *Calibration standards*.—Fresh calibration standards should be prepared every day, or as needed.

(*1*) Dilute the multi-element stock standard solutions into 50 mL precleaned autosampler vials with 5% HNO_3 in such a manner as to create a calibration curve. The lowest calibration standard (STD 1) should be equal to or less than the limit of quantitation (LOQ) when recalculated in units specific to the reported sample results.

(*2*) *See* Table **2015.01A** for recommended concentrations for the calibration curve.

(**g**) *Initial calibration verification (ICV) solution*.—Made up from second source standards in order to verify the validity of the calibration curve.

(**h**) *Calibration solutions*.—Daily optimization, tuning, and dual detector calibration solutions, as needed, should be prepared and analyzed per the instrument manufacturer's suggestions.

(**i**) *Certified Reference Materials (CRMs)*.—CRMs should preferably match the food matrix type being analyzed and contain the elements of interest at certified concentrations above the LOQ. Recommended reference materials include NIST SRM 1568a (Rice Flour), NIST SRM 1548a (Typical Diet), NRCC CRM DORM-3 (Dogfish Muscle), and NIST SRM 2976 (Mussel Tissue).

(**j**) *Spiking solution*.—50 mg/L Au and Lu in 5% (v/v) HNO_3. Prepared from single-element standards.

D. Contamination and Interferences

(**a**) Well-homogenized samples and small reproducible aliquots help minimize interferences.

(**b**) *Contamination*.—(*1*) Contamination of the samples during sample handling is a great risk. Extreme care should be taken to avoid this. Potential sources of contamination during sample handling include using metallic or metal-containing homogenization equipment, laboratory ware, containers, and sampling equipment.

(*2*) Contamination of samples by airborne particulate matter is a concern. Sample containers must remain closed as much as possible. Container lids should only be removed briefly and in a clean environment during sample preservation and processing, so that exposure to an uncontrolled environment is minimized.

(**c**) *Laboratory*.—(*1*) All laboratory ware (including pipet tips, ICP-MS autosampler vials, sample containers, extraction apparatus, and reagent bottles) should be tested for the presence of the metals of interest. If necessary, the laboratory ware should be acid-cleaned, rinsed with DIW, and dried in a Class 100 laminar flow clean hood.

(*2*) All autosampler vials should be cleaned by storing them in 2% (v/v) HNO_3 overnight and then rinsed three times with DIW. Then dry vials in a clean hood before use. Glass volumetric flasks should be soaked in about 5% HNO_3 overnight prior to use.

(*3*) All reagents used for analysis and sample preparation should be tested for the presence of the metals of interest prior to use in the laboratory. Due to the ultra-low detection limits of the method, it is imperative that all the reagents and gases be as low as possible in the metals of interest. It is often required to test several different sources of reagents until an acceptable source has been found. Metals contamination can vary greatly from lot to lot, even when ordering from the same manufacturer.

(*4*) Keep the facility free from all sources of contamination for the metals of interest. Replace laminar flow clean hood HEPA filters with new filters on a regular basis, typically once a year, to reduce airborne contaminants. Metal corrosion of any part of the facility should be addressed and replaced. Every piece of apparatus that is directly or indirectly used in the processing of samples should be free from contamination for the metals of interest.

(**d**) *Elemental interferences*.—Interference sources that may inhibit the accurate collection of ICP-MS data for trace elements are addressed below.

(*1*) *Isobaric elemental interferences*.—Isotopes of different elements that form singly or doubly charged ions of the same *m/z* and cannot be resolved by the mass spectrometer. Data obtained with isobaric overlap must be corrected for that interference.

(*2*)—*Abundance sensitivity*.--Occurs when part of an elemental peak overlaps an adjacent peak. This often occurs when measuring a small *m/z* peak next to a large *m/z* peak. The abundance sensitivity is affected by ion energy and quadrupole operating pressure. Proper optimization of the resolution during tuning will minimize the potential for abundance sensitivity interferences.

(*3*) *Isobaric polyatomic interferences*.—Caused by ions, composed of multiple atoms, which have the same *m/z* as the isotope of interest, and which cannot be resolved by the mass spectrometer. These ions are commonly formed in the plasma or the interface system from the support gases or sample components. The objective of IRT is to remove these

Table 2015.01B. Recommended isotopes for analysis

Element	Isotope, amu	Isotopic abundance, %	Potential interferences
Cd	111	13	MoO^+
	114	29	MoO^+, Sn^+
Hg	200	23	WO^+
	202	30	WO^+
Pb[a]	Sum of 206, 207, and 208	99	OsO^+

[a] Allowance for isotopic variability of lead isotopes.

interferences, making the use of correction factors unnecessary when analyzing an element in DRC mode. Elements not determined in DRC mode can be corrected by using correction equations in the ICP-MS software.

(**e**) *Physical interferences.*—(*1*) Physical interferences occur when there are differences in the response of the instrument from the calibration standards and the samples. Physical interferences are associated with the physical processes that govern the transport of sample into the plasma, sample conversion processes in the plasma, and the transmission of ions through the plasma-mass spectrometer interface.

(*2*) Physical interferences can be associated with the transfer of solution to the nebulizer at the point of nebulization, transport of aerosol to the plasma, or during excitation and ionization processes in the plasma. High levels of dissolved solids in a sample can result in physical interferences. Proper internal standardization (choosing internal standards that have analytical behavior similar to the associating elements) can compensate for many physical interferences.

(**f**) *Resolution of interferences.*—(*1*) For elements that are subject to isobaric or polyatomic interferences (such as As), it is advantageous to use the DRC mode of the instrument. This section specifically describes a method of using IRT for interference removal for As using a PerkinElmer DRC II and oxygen as the reaction gas. Other forms of IRT may also be appropriate.

(*a*) Arsenic, which is monoisotopic, has an *m/z* of 75 and is prone to interferences from many sources, most notably from chloride (Cl), which is common in many foods (e.g., salt). Argon (Ar), used in the ICP-MS plasma, forms a polyatomic interference with Cl at *m/z* 75 [$^{35}Cl + {}^{40}Ar = {}^{75}(ArCl)$].

(*b*) When arsenic reacts with the oxygen in the DRC cell, $^{75}As^{16}O$ is formed and measured at *m/z* 91, which is free of most interferences. The potential ^{91}Zr interference is monitored for in the following ways: ^{90}Zr and ^{94}Zr are monitored for in each analytical run, and if a significant Zr presence is detected, then $^{75}As^{16}O$ measured at *m/z* 91 is evaluated against the ^{75}As result. If a significant discrepancy is present, then samples may require analysis using alternative IRT, such as collision cell technology (helium mode).

(*c*) Instrument settings used (for PerkinElmer DRC II): DRC settings for $^{91}(AsO)$ and ^{103}Rh include an RPq value of 0.7 and a cell gas flow rate of 0.6 L/min. Cell conditions, especially cell gas flow rates, may be optimized for specific analyte/matrix combinations, as needed. In such cases, the optimized methods will often have slightly different RPq and cell gas flow values.

(*2*) For multi-isotopic elements, more than one isotope should be measured to monitor for potential interferences. For reporting purposes, the most appropriate isotope should be selected based on review of data for matrix interferences and based on the sensitivity (or relative abundance) of each isotope. The table below lists the recommended isotopes to measure. Low abundance isotopes are not recommended for this method as it is specifically applicable for ultra-low level concentrations (8–10 ppb LOQs). *See* Table **2015.01B**.

(**g**) *Memory effects*.—Minimize carryover of elements in a previous sample in the sample tubing, cones, torch, spray chamber, connections, and autosampler probe by rinsing the instrument with a reagent blank after samples high in metals concentrations are analyzed. Memory effects for Hg can be minimized through the addition of Au to all standard, samples, and quality control (QC) samples.

E. Sample Handling and Storage

(**a**) Food and beverage samples should be stored in their typical commercial storage conditions (either frozen, refrigerated, or at room temperature) until analysis. Samples should be analyzed within 6 months of preparation.

(**b**) If food or beverage samples are subsampled from their original storage containers, ensure that containers are free from contamination for the elements of concern.

F. Sample Preparation

(**a**) Weigh out sample aliquots (typically 0.25 g of as-received or wet sample) into microwave digestion vessels.

(**b**) Add 4 mL of concentrated HNO_3 and 1 mL of 30% hydrogen peroxide (H_2O_2) to each digestion vessel.

(**c**) Add 0.1 mL of the 50 mg/L Au + Lu solution to each digestion vessel.

(**d** Cap the vessels securely (and insert into pressure jackets, if applicable). Place the vessels into the microwave system according to the manufacturer's instructions, and connect the appropriate temperature and/or pressure sensors.

(**e**) Samples are digested at a minimum temperature of 190°C for a minimum time of 10 min. Appropriate ramp times and cool down times should be included in the microwave program, depending on the sample type and model of microwave digestion system. Microwave digestion is achieved using temperature feedback control. Microwave digestion programs will vary depending on the type of microwave digestion system used. When using this mechanism for achieving performance-based digestion targets, the number of samples that may be simultaneously digested may vary. The number will depend on the power of the unit, the number of vessels, and the heat loss characteristics of the vessels. It is essential to ensure that all vessels reach at least 190°C and be held at this temperature for at least 10 min. The monitoring of one vessel as a control for the batch/carousel may not accurately reflect the temperature in the other vessels, especially if the samples vary in composition and/or sample mass. Temperature measurement and control will depend on the particular microwave digestion system.

(*1*) *Note*: a predigestion scheme for samples that react vigorously to the addition of the acid may be required.

(*2*) The method performance data presented in this method was produced using a Berghof Speedwave 4 microwave digestion system, with the program listed in Table **2015.01C** (steps 1 and 2 are a predigestion step).

(*3*) Equivalent results were achieved using the program listed

Table 2015.01C. Digestion program for Berghof Speedwave 4 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	145	1	1
2	50	1	1
3	145	1	1
4	170	1	10
5	190	1	10

in Table **2015.01D** on a CEM MARS 6 microwave digestion system using the 40-position carousel and 55 mL Xpress digestion vessels.

(*4*) For infant formula samples, the program described in Table **2015.01E** has been shown to work effectively.

(**f**) Allow vessels to cool to room temperature and slowly open. Open the vessels carefully, as residual pressure may remain and digestate spray is possible. Pour the contents of each vessel into an acid-cleaned 50 mL HDPE centrifuge tube and dilute with DIW to a final volume of 20 mL.

(**g**) Digestates are diluted at least 4x prior to analysis with the 1% (v/v) HNO_3 diluent. When the metals concentration of a sample is unknown, the samples may be further diluted or analyzed using a total quantification method prior to being analyzed with a comprehensive quantitative method. This protects the instrument and the sample introduction system from potential contamination and damage.

(**h**) Food samples high in calcium carbonate ($CaCO_3$) will not fully digest. In such cases, the CRM can be used as a gauge for an appropriate digestion time.

(**i**) QC samples to be prepared with the batch (a group of samples and QC samples that are prepared together) include a minimum of three method blanks, duplicate for every 10 samples, matrix spike/matrix spike duplicate (MS/MSD) for every 10 samples, blank spike, and any matrix-relevant CRMs that are available.

G. Procedure

(**a**) *Instrument startup*.—(*1*) Instrument startup routine and initial checks should be performed per manufacturer recommendations.

(*2*) Ignite the plasma and start the peristaltic pump. Allow plasma and system to stabilize for at least 30 min.

(**b**) *Optimizations*.—(*1*) Perform an optimization of the sample introduction system (e.g., *X*-*Y* and *Z* optimizations) to ensure maximum sensitivity.

(*2*) Perform an instrument tuning or mass calibration routine whenever there is a need to modify the resolution for elements, or monthly (at a minimum), to ensure the instrument's quadrupole mass filtering performance is adequate. Measured masses should be ±0.1 amu of the actual mass value, and the resolution (measured peak width) should conform to manufacturer specifications.

Table 2015.01D. Digestion program for CEM MARS 6 microwave

Step	Temp., °C	Ramp, min	Hold, min
1	190	20	10
2	Cool down	NA	10

Table 2015.01E. Digestion program for infant formula

Step	Temp., °C	Ramp, min	Hold, min
1	180	20	20
2	Cool down	NA	20
3	200	20	20
4	Cool down	NA	20

(*3*) Optimize the nebulizer gas flow for best sensitivity while maintaining acceptable oxide and double-charged element formation ratios.

(*4*) Perform a daily check for instrument sensitivity, oxide formation ratios, double-charged element formation ratios, and background. If the performance check is not satisfactory, additional optimizations (a "full optimization") may be necessary.

(**c**) *Internal standardization and calibration*.—(*1*) Following precalibration optimizations, prepare and analyze the calibration standards prepared as described in **C**(**e**).

(*2*) Use internal standardization in all analyses to correct for instrument drift and physical interferences. Refer to **D**(**e**)(*2*). Internal standards must be present in all samples, standards, and blanks at identical concentrations. Internal standards can be added using a second channel of the peristaltic pump to produce a responses that is clear of the pulse-to-analog detector interface.

(*3*) Multiple isotopes for some analytes may be measured, with only the most appropriate isotope (as determined by the analyst) being reported.

(*4*) Use IRT for the quantification of As using the Rh internal standard.

(**d**) *Sample analysis*.—(*1*) Create a method file for the ICP-MS.

(*2*) Enter sample and calibration curve information into the ICP-MS software.

(*3*) Calibrate the instrument and ensure the resulting standard recoveries and correlation coefficients meet specifications (**H**).

(*4*) Start the analysis of the samples.

(*5*) Immediately following the calibration, an initial calibration blank (ICB) should be analyzed. This demonstrates that there is no carryover of the analytes of interest and that the analytical system is free from contamination.

(*6*) Immediately following the ICB, an ICV should be analyzed. This standard must be prepared from a different source than the calibration standards.

(*7*) A minimum of three reagent/instrument blanks should be analyzed following the ICV. These instrument blanks can be used to assess the background and variability of the system.

(*8*) A continuing calibration verification (CCV) standard should be analyzed after every 10 injections and at the end of the run. The CCV standard should be a mid-range calibration standard.

(*9*) An instrument blank should be analyzed after each CCV (called a continuing calibration blank, or CCB) to demonstrate that there is no carryover and that the analytical system is free from contamination.

(*10*) Method of Standard Additions (MSA) calibration curves may be used any time matrix interferences are suspected.

(*11*) Post-preparation spikes (PS) should be prepared and analyzed whenever there is an issue with the MS recoveries.

(**e**) Export and process instrument data.

H. Quality Control

(**a**) The correlation coefficients of the weighted-linear calibration curves for each element must be ≥0.995 to proceed with sample analysis.

(**b**) The percent recovery of the ICV standard should be 90–110% for each element being determined.

(**c**) Perform instrument rinses after any samples suspected to be high in metals, and before any method blanks, to ensure baseline sensitivity has been achieved. Run these rinses between all samples in the batch to ensure a consistent sampling method.

(**d**) Each analytical or digestion batch must have at least three preparation (or method) blanks associated with it if method blank correction is to be performed. The blanks are treated the same as the samples and must go through all of the preparative steps. If method blank correction is being used, all of the samples in the batch should be corrected using the mean concentration of these blanks. The estimated method detection limit (EMDL) for the batch is equal to 3 times the standard deviation (SD) of these blanks.

(**e**) For every 10 samples (not including quality control samples), a matrix duplicate (MD) sample should be analyzed. This is a duplicate of a sample that is subject to all of the same preparation and analysis steps as the original sample. Generally, the relative percent difference (RPD) for the replicate should be ≤30% for all food samples if the sample concentrations are greater than 5 times the LOQ. RPD is calculated as shown below. An MSD may be substituted for the MD, with the same control limits.

$$RPD = 200 \times \frac{|S1 - S2|}{S1 + S2}$$

where S1 = concentration in the first sample and S2 = concentration in the duplicate.

(**f**) For every 10 samples (not including quality control samples), an MS and MSD should be performed. The percent recovery of the spikes should be 70–130% with an RPD ≤30% for all food samples.

(*1*) If the spike recovery is outside of the control limits, an MSA curve that has been prepared and analyzed may be used to correct for the matrix effect. Samples may be corrected by the slope of the MSA curve if the correlation coefficient of the MSA curve is ≥0.995.

(*a*) The MSA technique involves adding known amounts of standard to one or more aliquots of the processed sample solution. This technique attempts to compensate for a sample constituent that enhances or depresses the analyte signal, thus producing a different slope from that of the calibration standards. It will not correct for additive interferences which cause a baseline shift.

(*b*) The best MSA results can be obtained by using a series of standard additions. To equal volumes of the sample are added a series of standard solutions containing different known quantities of the analyte(s), and all solutions are diluted to the same final volume. For example, addition 1 should be prepared so that the resulting concentration is approximately 50% of the expected concentration of the native sample. Additions 2 and 3 should be prepared so that the concentrations are approximately 100% and 150%, respectively, of the expected native sample concentration. Determine the concentration of each solution and then plot on the vertical axis of a graph, with the concentrations of the known standards plotted on the horizontal axis. When the resulting line is extrapolated to zero absorbance, the point of interception of the abscissa is calculated MSA-corrected concentration of the analyte in the sample. A linear regression program may be used to obtain the intercept concentration.

(*c*) For results of the MSA technique to be valid, take into consideration the following limitations:

(*i*) The apparent concentrations from the calibration curve must be linear (0.995 or greater) over the concentration range of concern.

(*ii*) The effect of the interference should not vary as the ratio of analyte concentration to sample matrix changes, and the MSA curve should respond in a similar manner as the analyte.

(*2*) If the sample concentration levels are sufficiently high, the sample may be diluted to reduce the matrix effect. Samples should be diluted with the 1% (v/v) HNO_3 diluent. For example, to dilute a sample by a 10x dilution factor, pipette 1 mL of the digested sample into an autosampler vial, and add 9 mL of the 1% (v/v) HNO_3 diluent. MS/MSD sets should be performed at the same dilution factor as the native sample.

(*3*) Spike at 1–10 times the level of a historical sample of the same matrix type, or, if unknown, spike at 1–5 times a typical value for the matrix. Spiking levels should be no lower than 10 times the LOQ.

(**g**) Percent recoveries of the CRMs should be 75–125% of their certified value.

(**h**) Percent recoveries of the CCV standards should be within 85–115%. Sample results may be CCV-corrected using the mean recovery of the bracketing CCVs. This should only be done after careful evaluation of the data. The instrument should show a trending drift of CCV recoveries and not just a few anomalous outliers.

(**i**) CCBs should be monitored for the effects of carryover and for possible system contamination. If carryover of the analyte at levels greater than 10 times the MDL is observed, the sample results may not be reportable.

(**j**) Absolute response of any one internal standard should not vary from the original response in the calibration blank by more than 60–125%. Some analytical samples, such as those containing concentrations of the internal standard and tissue digestates, can have a serious effect on the internal standard intensities, but this does not necessarily mean that the analytical system is out of control. In some situations, it is appropriate to reprocess the samples using a different internal standard monitored in the analysis. The data should be carefully evaluated before doing this.

(**k**) The recovery of the Lu that was spiked into the sample preparation prior to digestion should be evaluated to assess any potential loss of analyte during the process. The concentration of Lu in the sample preparation is 0.25 mg/L, and for samples diluted 4x at the instrument, this is equivalent to 62.5 µg/L at the instrument (if samples are diluted more than 4x, this must be taken into account). The Lu recovery should be no less than 75% of the original spiked concentration.

(**l**) Refer to Table **2015.01F** for a summary of all recommended quality control samples, minimum frequency at which they are to be analyzed, acceptance criteria for each, and

Table 2015.01F. Summary of quality control samples

QC sample	Measure	Minimum frequency	Acceptance criteria	Corrective action
Calibration standards	Linearity of the calibration curve	Analyzed once per analytical day	Correlation coefficient ≥0.995, 1st standard ≤MRL, low standard recovery = 75–125%, all other standard recoveries = 80–120%	Reanalyze suspect calibration standard. If criteria still not met, then re-prepare standards and recalibrate the instrument.
Internal standards	Variation in sample properties between samples and standards	Each standard, blank, and sample is spiked with internal standard	60–125% recovery compared to calibration blank	If the responses of the internal standards in the following CCB are within the limit, rerun the sample at an additional 2x dilution. If not, then samples must be reanalyzed with a new calibration.
Lu digestion check spike	Assessment of potential loss during digestion	Added to every digested samples	Recovery ≥75%	Re-prepare the sample
Initial calibration verification (ICV)	Independent check of system performance	One following instrument calibration	Recovery = 90–110%	Correct problem prior to continuing analysis. Recalibrate if necessary.
Continuing calibration verification (CCV)	Accuracy	At beginning and end of analysis and one per 10 injections	Recovery = 85–115%	Halt analysis, correct problem, recalibrate, and reanalyze affected samples
Method blanks (MB)	Contamination from reagents, lab ware, etc.	Minimum of three per batch	Mean ≤ MRL; SD ≤ MDL or MBs <1/10th sample result	Determine and eliminate cause of contamination. Affected samples must be re-prepared and reanalyzed.
Method duplicates (MD)	Method precision within a given matrix	Minimum of one per 10 samples	RPD ≤ 30% or ±2x LOQ if results ≤5x LOQ	If RPD criteria not met, then sample may be re-prepared and reanalyzed, but this is not required. Sample matrix may be inhomogeneous. A post-digestion duplicate (PDD) can be analyzed to evaluate instrument precision.
Matrix spikes/matrix spike duplicates (MS/MSD)	Method accuracy and precision within a given matrix	Minimum of one per 10 samples	Recovery = 70–130% and RPD ≤ 30%	If RPD >30%, results must be qualified
Post-preparation spike (PS)	Check for matrix interference	When required (samples spiked too low/high, dilution test fails, etc.)	Recovery = 75–125%	Analyze samples using MSA or results flagged accordingly
Laboratory fortified blank (LFB) or blank spike (BS)	Method accuracy	Minimum of one per batch	Recovery = 75–125%	If LFB recovery is outside of the control limit, then batch must be re-prepared and reanalyzed
Certified Reference Material (CRM)	Method accuracy	Must be matrix-matched to samples; minimum of one per batch	Recovery = 75–125% unless limits set by CRM manufacturer are greater or element/CRM specific limits have been established	If CRM true value is ≥5x the LOQ and recovery is outside of the control limit, then batch must be re-prepared and reanalyzed

Table 2015.01G. Method blank results and LOD/LOQ, µg/kg

Method blanks	^{91}(AsO)	^{111}Cd	^{114}Cd	Pb	^{200}Hg	^{202}Hg
MB-01	2.83	0.229	0.270	1.90	1.61	0.95
MB-02	1.48	–0.088	0.270	0.14	1.48	1.13
MB-03	1.80	0.007	0.115	0.13	0.76	0.25
MB-04	1.03	0.154	0.288	0.12	1.46	0.33
MB-05	1.43	0.010	0.259	1.84	1.28	0.27
MB-06	1.07	0.105	0.096	3.02	0.87	0.76
MB-07	2.31	–0.002	0.297	2.67	0.89	0.44
MB-08	1.20	0.285	0.200	4.24	0.55	0.28
MB-09	1.05	0.002	0.182	0.09	0.96	0.25
MB-10	2.12	0.047	0.150	0.19	0.71	0.02
MB-11	2.09	–0.145	0.226	0.12	0.64	0.57
MB-12	1.44	0.037	0.165	0.18	0.45	0.50
MB-13	0.70	–0.122	0.160	0.17	0.81	0.19
MB-14	1.12	–0.001	0.074	0.14	0.85	0.21
MB-15	2.33	0.097	0.207	0.11	0.18	0.17
MB-16	1.53	–0.117	0.146	0.16	1.33	1.09
MB-17	1.79	–0.070	0.180	0.03	3.46	2.19
MB-18	1.90	0.049	0.115	0.06	3.30	2.36
MB-19	1.18	0.043	0.224	0.39	4.01	2.78
MB-20	1.24	–0.060	0.199	0.07	0.99	0.56
MB-21	0.92	0.165	0.120	0.03	0.73	0.33
MB-22	1.69	0.005	0.186	0.09	0.60	0.25
MB-23	2.13	0.171	0.152	0.08	0.41	–0.23
SD	0.54	0.113	0.063	1.18	1.01	0.77
LOD	1.6	0.50[a]	0.50[a]	3.5	3.0	2.3
LOQ	3.3	1.60[a]	1.60[a]	7.1	6.0	4.6

[a] Adjusted to conform to lowest calibration point.

appropriate corrective action if the acceptance criteria are not met.

I. Method Performance

(**a**) Limit of detection (LOD) and LOQ were determined through the analysis of 23 method blanks (*see* Table **2015.01G**). LOD was calculated as 3 times the SD of the results of the blanks, and LOQ was calculated as 2 times the value of the LOD, except where the resulting LOQ would be less than the lowest calibration point, in which case LOQ was elevated and set at the lowest calibration point and LOD was calculated as 1/3 of the LOQ. All LOQs achieved are ≤10 µg/kg for all food matrices and ≤8 µg/kg for liquid matrices, such as infant formula.

(**b**) Sample-specific LOQs for several matrices, based on LOQs determined by the default method, and adjusted for changes in sample mass for particular samples, are shown in Table **2015.01H**. Values have been rounded up to the nearest part-per-billion.

Table 2015.01H. Sample-specific LOQs

	LOQ, µg/kg (as received)			
Sample	As	Cd	Pb	Hg
Infant formula	2	1	4	3
Chocolate	4	2	8	6
Rice flour	4	2	8	6
Fruit juice	1	1	2	2

Table 2015.01I. Recoveries for numerous relevant CRMs

Certified Reference Material	As, %	Cd, %	Pb, %	Hg, %
DOLT-4 Dogfish Liver	104	97	87	114
DORM-3 Fish Protein	105	109	94	114
DORM-4 Fish Protein	105	91	91	81
NIST 1548a Typical Diet	103	95	113	NA
NIST 1568a Rice Flour	98	99	NA	NA
NIST 1946 Lake Superior Fish Tissue	119	NA	NA	101
TORT-2 Lobster Hepatopancreas	109	104	95	116
TORT-3 Lobster Hepatopancreas	113	89	86	86

Table 2015.01J. AOAC SMPR 2012.007 (ref. 1)

Concn range, µg/kg	Repeatability, %	Reproducibility, %	Recovery, %
LOQ–100	15	32	60–115
100–1000	11	16	80–115
>1000	7.3	8	80–115

(**c**) Numerous relevant CRMs were analyzed to establish method accuracy. Example percent recoveries are provided in Table **2015.01I** (recoveries have been omitted for CRMs that do not provide a certified value or if the certified value is less than the LOQ).

(**d**) *Standard Method Performance Requirements*[SM] (AOAC SMPR **2012.007**; 1) for repeatability, reproducibility, and recovery for the method are shown in the Table **2015.01J**. *See* Appendix A (Appendix A is available on the *J. AOAC Int.* website as supplemental material, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac) for detailed method performance information supporting acceptance of the method.

(**e**) *See* Appendix A for detailed method performance information supporting acceptance of the method. Method validation samples were prepared and analyzed for all applicable matrices. In general, all SMPR criteria were met for As, Cd, Hg, and Pb in the matrices apple juice, infant formula, cocoa powder, and rice flour.

References

(1) *J. AOAC Int.* **96**, 704(2013) DOI: 10.5740/jaoac.int.2012.007

AOAC Official Method 2015.02
Sodium Monofluoroacetate in Dairy Powders
Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)
First Action 2015

(The method has been validated in cow, goat, and sheep dairy powders, and dairy powder formulations. It can be used for other similar matrixes, including liquid milk, provided that it is demonstrated that the method performance values are met.)

Specific hazards.—*Hydrochloric acid*.—Wear personal protective equipment (PPE) and handle in a fume cupboard.

Sulfuric acid.—Wear PPE and handle in a fume cupboard.

Sodium fluoroacetate.—Wear PPE, including safety glasses and a dust mask, when weighing out the primary material.

Potassium hydroxide.—Wear PPE and handle in a fume cupboard; avoid inhalation of dust.

3-Nitroaniline.—Wear PPE, including safety glasses; avoid inhalation.

Phosphoric acid.—Wear PPE and handle in a fume cupboard.

Sodium fluoroacetate (Figure **2015.02A**) is a rodenticide used in New Zealand to control rats, possums, and rabbits. It is commonly known by its original registration number as compound 1080, but may also be known as sodium monofluoroacetate and fluoroacetic acid sodium salt.

A. Principle

Samples are dissolved in water and extracted into acetone to allow precipitation of proteins. After centrifugation, the solutions are passed through an anion exchange column and eluted with acid to give free fluoroacetic acid. This acid is converted to 2-fluoro-3'-nitroacetanilide via a carbodiimide-mediated amide coupling reaction. The derivative is then subjected to SPE cleanup, eluting with *t*-butyl methyl ether (TBME)–*n*-hexane, concentrated and quantified by LC-MS/MS using derivatized isotopically substituted sodium fluoroacetate as an internal standard. The method reports the analyte as fluoroacetic acid.

B. Apparatus

Note: Where specific equipment is listed, other brands or models may be used provided that they have equivalent performance.

Laboratory equipment.—

(**a**) *Air displacement pipets*.—5000 µL, with long tips.

(**b**) *Autosampler vials*.—2 mL with tapered glass inserts.

(**c**) *Balance*.—2 or 3 decimal top pan.

(**d**) *Balance*.—5 decimal place analytical.

(**e**) *Centrifuge*.—Capable of centrifuging 15 and 50 mL tubes at 4200 × *g* RCF.

(**f**) *Centrifuge tubes*.—15 and 50 mL, tapered polypropylene.

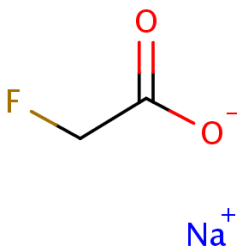

Figure 2015.02A. Sodium fluoroacetate.

Table 2015.02A. Fortification of recovery samples

Tube No.	Name	Volume, µL WS3	WS2	WS3	Concn fluoroacetic acid, µg/kg
1[a]	Matrix standard	0[a]	0[a]	0[a]	5[a]
2	Recovery 1	0	0	40	0
3	Recovery 2	25	0	40	0.1
4	Recovery 3	125	0	40	0.5
5	Recovery 4	0	25	40	1
6	Recovery 5	0	125	40	5
7	Reagent blank	0	0	40	0

[a] The matrix standard is fortified at step **F**(**b**)(*9*).

(**g**) *Extraction cartridges*.—Oasis HLB 60 mg.

(**h**) *Glass reservoirs*.—Ground glass, 19/26 joint, approximately 20 mL.

(**i**) *Laboratory glassware*.—Measuring cylinders, volumetric flasks, and beakers.

(**j**) *Multi-step dispenser with appropriate tips*.—Eppendorf Stream or equivalent.

(**k**) *Nitrogen blow-down*.—Capable of maintaining a temperature 40 ± 10°C.

(**l**) *pH meter*.

(**m**) *Polypropylene SPE reservoirs*.—10 mL, with adapters.

(**n**) *Positive displacement pipets*.—25, 100, 250, and 1000 µL, with tips.

(**o**) *Refrigerator and freezer.*

(**p**) *Resin chromatography column*.—10 mL polypropylene (Bio-Rad).

(**q**) *Shaker*.—Reciprocating bench top.

(**r**) *Ultrasonic bath*.

(**s**) *Vacuum manifold for SPE cartridges*.—With stopcocks.

(**t**) *Vortex mixer*.

(**u**) *Water bath*.—Maintained at 40 ± 2°C.

Analytical instrumentation.—

(**v**) *LC-MS/MS instrument*.—AB Sciex 5500 QTRAP coupled with Agilent 1290 Series HPLC.

(**w**) *HPLC guard column*.—Phenomenex Security C18, 4 × 2 mm.

(**x**) *HPLC column*.—Agilent XDB-C18, 100 × 4.6 mm, 1.8 µm.

C. Reagents

All reagents and chemicals must be of such a grade that they do not interfere with the analytical process.

Chemicals.—

(**a**) *Acetone*.—Pesticide grade.

(**b**) *Acetonitrile*.—Pesticide grade.

(**c**) *AG 1-X8 resin*.—100–200 mesh chloride form, ACS reagent grade.

(**d**) *Ammonium acetate*.—ACS reagent grade.

(**e**) *Deionized water*.—Laboratory purified, ≥18 ΩM.

(**f**) *Hydrochloric acid*.—Concentrated, ACS reagent grade; 37–38%.

(**g**) *Methanol*.—Pesticide grade.

(**h**) *n-Hexane*.—Pesticide grade.

(**i**) *Phosphoric acid*.—Concentrated, ACS reagent grade.

(**j**) *Potassium dihydrogen phosphate*.—ACS reagent grade.

(**k**) *Potassium hydroxide*.—ACS reagent grade.

Table 2015.02B. Identification parameters for compounds analyzed as negative ions

Compound (3-nitroaniline derivatives of analyte and internal standard)	Expected retention time, min	Molecular ion (Q1)	Product ion (Q3)	Dwell, ms	DP, V	CE, eV	CXP, V
2-Fluoro-3′-nitroacetanilide	2.06	196.931	122.000	50	–120	–24	–17
		196.931	146.900	50	–120	–22	–23
		196.931	117.800	50	–120	–28	–17
1,2-^{13}C-2,2-D-2-fluoro-3′-nitroacetanilide	2.06	201.001	134.900	50	–115	–30	–21
		201.001	45.900	50	–115	–22	–23

(**l**) *Sodium sulfate, anhydrous*.—ACS reagent grade.

(**m**) *Sodium hydrogen carbonate*.—ACS reagent grade.

(**n**) *Sulfuric acid*.—Concentrated, ACS reagent grade.

(**o**) *TBME*.—Pesticide grade.

(**p**) *1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC)*.—ACS reagent grade.

(**q**) *3-Nitroaniline*.—ACS reagent grade.

Solutions.—

(**a**) *5 M hydrochloric acid (2000 mL)*.—(*Caution*: Preparation of solution should be performed inside a fume cupboard.) Into a 2000 mL volumetric flask add 800 mL deionized water. To this add 832 mL of 37–38% (12 M) concentrated hydrochloric acid. Mix and allow to cool. Bring to volume with deionized water. Store at room temperature.

(**b**) *0.2 M hydrochloric acid (2000 mL)*.—Into a 2000 mL volumetric flask add 800 mL deionized water. To this add 80 mL of 5 M hydrochloric acid. Mix and allow to cool, and bring to volume with deionized water. Store at room temperature.

(**c**) *2 M potassium hydroxide (2000 mL)*.—(*Caution*: Preparation of solution should be performed inside a fume cupboard.) Place 1600 mL deionized water in a 2000 mL beaker and place on a magnetic stirrer with follower. Weigh 224.4 g potassium hydroxide into a 500 mL beaker. Add the potassium hydroxide, a few pellets at a time, to the stirred solution. Do not allow the temperature to rise above warm to the touch. When all the pellets have dissolved, allow the solution to cool and then transfer quantitatively through a glass funnel into a 2000 mL measuring cylinder and bring to volume with deionized water. Store at room temperature.

(**d**) *20 mg/mL 3-nitroaniline (100 mL)*.—(*Caution*: Wear gloves when working with this chemical.) Weigh 2.0 g aliquots of 3-nitroaniline into 100 mL Schott bottles and cap tightly. Store at room temperature.

To one preweighed bottle of 3-nitroaniline add 100 mL acetonitrile using a graduated measuring cylinder. This is sufficient for two batches of 36 sample tubes. Prepare fresh daily.

(**e**) *100 mg/mL EDAC (25 mL)*.—(*Caution*: Exposure to moisture degrades this reagent.) Preweigh 2.5 g aliquots of EDAC into 50 mL polypropylene tubes and cap tightly. Store in a freezer at or below –10°C in a desiccated container.

To one preweighed tube of EDAC add 25 mL deionized water. Prepare fresh daily.

(**f**) *TBME–n-hexane (70 + 30, v/v; 2000 mL)*.—Measure 1400 mL TBME into a 2000 mL Schott bottle and add 600 mL *n*-hexane. Cap and mix. Store at room temperature.

(**g**) *Sulfuric acid in water (25%, v/v; 2000 mL)*.—(*Caution*: Preparation of solution should be performed inside a fume cupboard.) Add approximately 1200 mL deionized water to a 2 L volumetric flask followed by slow addition of 500 mL sulfuric acid. Mix and allow to cool to room temperature. Bring to 2 L volume and store in a Schott bottle. Store at room temperature.

(**h**) *0.05 M potassium dihydrogen phosphate, pH 2.3 (1000 mL)*.—Weigh 6.80 g potassium dihydrogen phosphate into a 500 mL beaker. Add 300 mL deionized water to dissolve the potassium dihydrogen phosphate and quantitatively transfer into a 1 L Schott bottle. Add a further 300 mL deionized water to the beaker to dissolve any remaining potassium dihydrogen phosphate, quantitatively transfer into the Schott bottle, make up to 1 L. Cap, mix, and adjust the pH to 2.3 ± 0.1 with concentrated phosphoric acid. Store at room temperature.

(**i**) *0.1 M sodium hydrogen carbonate (1000 mL)*.—Weigh 8.40 g sodium hydrogen carbonate into a 500 mL beaker. Add 300 mL deionized water to dissolve the sodium hydrogen carbonate and quantitatively transfer into a 1 L Schott bottle. Add a further 300 mL deionized water to the beaker to dissolve any remaining sodium hydrogen carbonate, quantitatively transfer into the Schott bottle, make up to 1 L, cap, and mix to ensure full solubility. Store at room temperature.

(**j**) *AG 1-X8 anion exchange resin*.—Before use, soak the AG 1-X8 anion exchange resin in deionized water for 18–24 h, and store in deionized water until use. Store in a refrigerator.

(**k**) *HPLC mobile phase A: 10 mM ammonium acetate in water (1000 mL)*.—Weigh 0.77 g ammonium acetate into a 1 L Schott bottle, followed by 1000 mL deionized water. Cap and mix to ensure full solubility. Store at room temperature.

(**l**) *HPLC mobile phase B: 10 mM ammonium acetate in 97% acetonitrile (1000 mL)*.—Weigh 0.77 g ammonium acetate into a 50 mL beaker. Use 30 mL deionized water to quantitatively transfer to a 1 L Schott bottle. Stir to fully dissolve and add 970 mL acetonitrile. Sonicate for 10 min to ensure full solubility. Store at room temperature.

D. Standards

(**a**) *Primary standards*.—Primary standards are stored in a refrigerator between 2–8°C in the dark. Subsequent solutions should be corrected for purity, moisture, and salt (if applicable). Wear appropriate PPE when weighing out the primary material.

(*1*) *Analytes*.—Sodium fluoroacetate (CAS No. 62-74-8).

(*2*) *Internal standard*.—$^{13}C_2D_2$ sodium fluoroacetate.

(**b**) *Secondary standards*.—(*1*) *Analytes*.—*Fluoroacetic acid (1000 mg/L)*.—Weigh approximately 12.9 mg sodium fluoroacetate into a calibrated 10 mL volumetric flask. Add deionized water, make to volume, and mix until solid is completely dissolved. Transfer to a 15 mL polypropylene screw cap test tube and cap tightly. Store in a freezer at less than –10°C. Calculate exact concentration correcting for purity, moisture (if applicable), and salt using Equation 1.

$$C = [(m \times P \times Mo)/V] \times [MW/MW_{(salt)}] \quad (1)$$

where C = concentration of standard solution in mg/L; m = exact mass of primary standard weighed in mg; P = purity of standard expressed as a decimal equal to % purity/100; Mo = additional purity correction for moisture/water (if applicable). Expressed as a decimal equal to (100 – % moisture)/100; V = volume of solution in L; MW = molecular weight of target analyte; $MW_{(salt)}$ = molecular weight of analyte as salt.

(*2*) *Internal standard.—*$^{13}C_2D_2$ *fluoroacetic acid (500 mg/L).*—Weigh approximately 7.2 mg (corrected for chemical and isotopic purity) of $^{13}C_2D_2$ labeled sodium fluoroacetate into a calibrated 10 mL volumetric flask. Add deionized water, make to volume, and mix until solid is completely dissolved. Transfer to a 15 mL polypropylene screw cap test tube and cap tightly. Store in the freezer at –10°C or below. Calculate exact concentration correcting for purity, moisture (if applicable), and salt using Equation 1.

(**c**) *Intermediate standards.—*(*1*) *Analyte.—Fluoroacetatic acid intermediate standard (50 mg/L).*—Dilute 2.5 mL (calculate exact volume based on concentration of secondary standard using Equation 2) of fluoroacetic acid secondary standard (1000 mg/L) to 50 mL with deionized water in a calibrated volumetric flask. Use a calibrated positive displacement pipet. Dispense aliquots of the standard into 15 mL polypropylene tubes for frozen storage. Store in the freezer at –10°C or below.

$$C_2 = (C_1 \times V_1)/V_2 \quad (2)$$

where C_2 = concentration of required diluted solution in mg/L; C_1 = concentration of high standard in mg/L; V_1 = volume of high standard required in mL; V_2 = total volume of diluted solution in mL.

(*2*) *Internal standard.—*$^{13}C_2D_2$ *fluoroacetic acid standard (50 mg/L).*—Dilute 2.5 mL (calculate exact volume based on concentration of secondary standard using Equation 2) of $^{13}C_2D_2$ fluoroacetic acid secondary standard (500 mg/L) to 25 mL with water in a calibrated volumetric flask. Use a calibrated positive displacement pipet. Dispense aliquots of the standard into 15 mL polypropylene tubes. Store in the freezer at –10°C or below.

(**d**) *Working solutions (WS).—*(*1*) *Analyte.—*(*a*) *WS1 (1.0 mg/L).*—Dilute 1000 µL fluoroacetic acid intermediate standard (50 mg/L) to 50 mL with deionized water in a calibrated volumetric flask. Dispense aliquots of the WS1 standard into 15 mL polypropylene tubes. Store in the freezer at –10°C or below. A thawed and opened tube WS1 can be stored for up to 2 months in a refrigerator between 2–8°C provided it is resealed and immediately refrigerated after each use.

(*b*) *WS2 (0.1 mg/L).*—Dilute 10 mL of WS1 (1.0 mg/L) to 100 mL with deionized water in a calibrated volumetric flask. Dispense aliquots of the WS2 standard into 15 mL polypropylene tubes. Store in the freezer at –10°C or below. A thawed and opened tube WS2 can be stored for up to 2 months in a refrigerator between 2–8°C provided it is resealed and immediately refrigerated after each use.

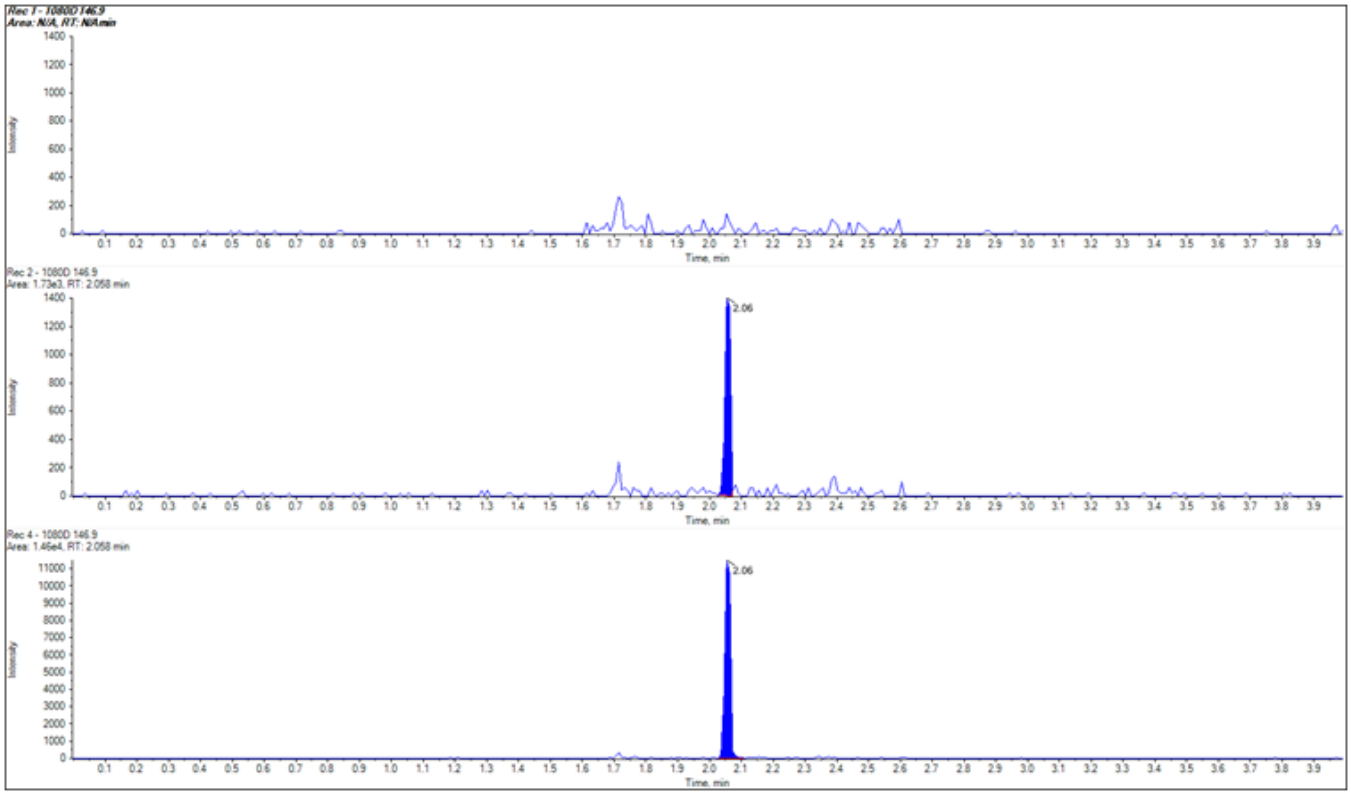

Figure 2015.02B. Chromatograms of confirmation ion for recovery 1 (matrix blank; top), recovery 2 (0.1 µg/kg, ≈LOQ; middle), and recovery 4 (1.0 µg/kg, LOR; bottom).

Table 2015.02C. HPLC solvent gradient

Time	% A (10 mM NH_4Ac in H_2O)	% B (10 mM NH_4Ac in 97% ACN)
0	80	20
2.50	0	100
3.00	0	100
3.01	80	20
4.00	80	20

(*c*) *WS3 (0.01 mg/L)*.—Dilute 500 µL of WS2 (0.1 mg/L) to 5 mL with deionized water in a calibrated volumetric flask. Transfer into a 15 mL polypropylene tube. Make fresh daily.

(*2*) *Internal standard.—Internal standard working solution (ISWS; 0.5 mg/L)*.—Dilute 1000 µL of $^{13}C_2D_2$ fluoroacetic acid intermediate standard (50 mg/L) to 100 mL with deionized water in a calibrated volumetric flask. Pipet aliquots of the ISWS into 15 mL polypropylene tubes. Store in the freezer at –10°C or below. A thawed and opened tube of ISWS can be stored for up to 2 months in a refrigerator between 2–8°C provided it is resealed and immediately refrigerated after each use.

E. Sampling and Sample Preparation

Preparation of test portion.—Accurately weigh 2.5 ± 0.03 g of room temperature sample into a labeled 50 mL polypropylene tube. In addition to the analytical samples, there are four recovery samples per batch, a reagent blank, and an extra blank for the matrix standard.

F. Procedure

(**a**) *Fortification*.—(*1*) *Analyte*.—Fortify the recoveries as shown in Table **2015.02A** using the working solutions prepared in **D**(**d**). *Note*: Do not add any WS to the matrix blank to be used for the matrix standard.

(*2*) *Internal standard*.—Add 40 µL ISWS to all unknown and recovery samples. *Note*: Do not add any ISWS to the matrix blank to be used for the matrix standard.

(*3*) Allow test portions to equilibrate for 10 min at room temperature.

(**b**) *Extraction*.—(*1*) Place the resin chromatography columns onto a vacuum manifold and fill with 1.4 (±0.2) mL resin. Add 2.5 mL deionized water above the resin bed and close stopcock. Fit suitable reservoirs above the columns.

(*2*) To each test portion add 5 mL water and briefly shake vigorously by hand, cap, and then shake tubes at medium speed on a reciprocating shaker for 5 min to dissolve. Variation to this procedure may be required for atypical matrixes.

(*3*) Add 10 mL acetone to each tube and briefly shake vigorously by hand followed by 2 min on a reciprocating shaker at medium speed.

(*4*) Centrifuge at 4200 × *g* RCF for 10 min.

(*5*) Carefully pour the top solvent layer into the reservoirs above the resin, taking care not to transfer any precipitate.

(*6*) Allow samples to pass through the resin columns under gravity or gentle vacuum, if required.

(*7*) After samples have passed through the resin columns, remove the reservoirs and wash the resin columns with 1 mL of 0.2 M hydrochloric acid. Close stopcock. Do not allow the resin to dry.

(*8*) Place 15 mL polypropylene tubes beneath each resin column. Elute samples with one 5 mL volume of 0.2 M hydrochloric acid at about 30 drops/min. Remove residual hydrochloric acid solution into the collecting tubes under vacuum.

(*9*) To the matrix standard tube only, add 125 µL WS2 and 40 µL ISWS, cap, and vortex mix.

(*10*) To all tubes add 1.25 mL of 20 mg/mL 3-nitroaniline and 0.25 mL of 100 mg/mL EDAC solution followed by 0.5 mL of 2 M potassium hydroxide and 1 mL of 0.05 M potassium dihydrogen phosphate buffer. Cap and mix.

(*11*) Place tubes in a 40 ± 2°C water bath for 20 min.

(*12*) Remove tubes and cool to room temperature.

(*13*) Set up a vacuum manifold with Oasis HLB, 60 mg, 3 mL cartridges.

(*14*) Condition the cartridge with 1 mL methanol. Close the stopcock when the methanol reaches the top frit.

(*15*) Load a portion of the derivatized extract onto the conditioned SPE cartridge.

(*16*) Place an adapter and 10 mL reservoir on top of the cartridge.

(*17*) Transfer the remaining derivatized extract into the reservoir and open the stopcock. Allow to drip slowly to waste at about 30–40 drops/min.

(*18*) When the extract has passed through the cartridge, remove the adapter and reservoir.

(*19*) Wash the cartridge with 2 mL 25% (v/v) sulfuric acid, 1 mL deionized water, 1 mL 0.1 M sodium hydrogen carbonate, and a further 2 mL deionized water to waste.

(*20*) Dry cartridge by applying full vacuum for 5 min.

(*21*) Place 15 mL polypropylene tubes beneath each SPE. Elute the derivatized extract with 2 × 2.5 mL TBME–*n*-hexane (70 + 30, v/v) into the tubes.

(*22*) Dry the cartridge by briefly applying a full vacuum.

(*23*) Check tubes for remaining water. There should be minimal water present. Presence of more than about 50 µL water would indicate inadequate vacuum.

Table 2015.02D. Instrument parameters for AB Sciex LC-MS/MS system

Parameter	Value
HPLC column	Agilent XDB-C18, 100 × 4.6 mm × 1.8 µm
Column temperature	60°C
Autosampler temperature	10°C
Flow rate	1 mL/min
Injection volume	5 µL
Run time	4 min
Ionization mode	Electrospray
Polarity	Negative
Curtain gas (CUR)	30 psi
Source temp. (TEM)	750°C
Ion source gas 1 (GS1)	60 psi
Ion source gas 2 (GS2)	60 psi
Ion spray voltage (IS)	–4500 V
Collision gas (CAD)	Medium (8)
Entrance potential (EP)	–10 V

Table 2015.02E. Performance values of analytes[a]

Compound	LOD, µg/kg	LOQ, µg/kg	LOR, µg/kg	Within-day CV	Between-day CV (WLR)	U (for 95% CI)	Recovery, % (SD)
Fluoroacetic acid	0.028	0.085	1.0[b]	8.8	9.1	18	97[c] (8.8)
$^{13}C_2$ D_2 Fluoroacetic acid	NA	NA	NA	NA	NA	NA	70[d] (12)

[a] LOD = Limit of detection; LOQ = limit of quantification; CV = coefficient of variation; WLR = within-laboratory reproducibility; U = uncertainty of measurement with a 95% confidence interval; SD = standard deviation.

[b] Limit of reporting (LOR) set according to New Zealand maximum permitted residue limits. *See* reference 1.

[c] Relative recovery.

[d] Absolute recovery.

(*24*) Add approximately 200 mg sodium sulfate, anhydrous, to each tube and vortex mix.

(*25*) Centrifuge at 2400 × *g* RCF for 1 min.

(*26*) Decant the supernatant into a clean 15 mL tapered, polypropylene tube.

(*27*) Evaporate the solvent to incipient dryness under nitrogen at 40 ± 10°C.

Note: Do not leave on heating block as excess heating may degrade derivatized analyte.

(*28*) Allow tubes to return to near room temperature and then redissolve residue in 150 µL acetonitrile.

(*29*) Vortex mix at low speed.

(*30*) Centrifuge at 2400 × *g* RCF for 1 min.

(*31*) Transfer clear solvent layer to a tapered insert in an autosampler vial, making sure not to transfer any solid and/or particulate matter. Cap firmly.

Note: Final extracts have been shown to be stable at least 5 days when stored in the freezer at –10°C or below.

(**c**) *Instrumental determination*.—(*1*) *Identification parameters*.—Identification parameters for the analysis of sodium fluoroacetic acid are given in Table **2015.02B**.

(*2*) *Analytical instrumentation*.—(*a*) *General*.—Agilent 1290 HPLC system coupled with a 5500 QTRAP Triple Quad Mass Spectrometer. The system is controlled by AB Sciex Analyst software. Peak integration is handled with AB Sciex MultiQuant Analysis software.

Note: *See* Figure **2015.02B** for exemplary chromatograms.

(*b*) *LC parameters*.—*See* Table **2015.02C** for HPLC solvent gradient.

(*i*) *Column*.—Agilent XDB-C18, 100 × 4.6 mm.

(*ii*) *Guard column*.—Phenomenex Security C18, 4 × 2 mm.

(*c*) *Mass spectrometer parameters*.—*See* Table **2015.02D** for full analytical parameters.

G. Calculations

Quantification of fluoroacetic acid is based on peak area. Matrix recoveries are used to generate calibration curves. An unknown peak that falls within the evaluation window (as calculated by recoveries and internal standard) is quantified from the appropriate calibration curve and the value tabulated, together with peak identification information. Each potential unknown is then manually assessed for the quality of identification by viewing integrated chromatograms and those of any qualifying ions.

$$C_u = RR/Sl$$

where C_u = concentration of unknown sample in µg/kg; *RR* = relative response of unknown sample; *Sl* = slope of calibration curve.

H. Method Performance and Quality Control

(**a**) *Reagent blank test*.—A reagent blank (deionized water) test is performed with each batch.

(**b**) *Matrix standard test*.—Performed with each batch according to Table **2015.02A**.

(**c**) *Matrix blank test (Recovery 1)*.—A matrix blank test is performed with each batch.

(**d**) *Matrix recovery test (recovery samples)*.—Performed with each batch according to Table **2015.02A**.

(**e**) *Certified reference materials (CRM)*.—No CRM is currently available. In practice, external checks of the method are performed by participation in interlaboratory calibration studies when available.

(**f**) *Performance values*.—Values found in Table **2015.02E** are calculated from the in-house single-laboratory validation (SLV) completed by AsureQuality Ltd.

(**g**) *Acceptance criteria*.—(*1*) *Individual sample acceptance criteria*.—The internal standard response for an individual sample should exceed 33% of the mean internal standard response of the recovery samples.

(*2*) *Batch acceptance criteria*.—Analyte relative recoveries for the recovery samples should be within 3 SD of the mean relative recovery established from control charts. Calibration curves should have a coefficient of determination $R^2 > 0.95$.

(*3*) *Positive sample acceptance criteria*.—Retention time acceptance criteria are given in Table **2015.02F**. Ion ratio acceptance limits are given in Table **2015.02G**.

Table 2015.02F. Relative retention time (RRT) and limits of acceptance

Compound (3-nitroaniline derivative of analyte)	Monitored compounds	RRT	Acceptance limit[a]
2-Fluoro-3′-nitroacetanilide	Analyte/internal standard	1.004[b]	RRT ± 2.5%

[a] *See* reference 2.

[b] Representative relative retention time. These values are indicative and should be measured for each individual batch.

Table 2015.02G. Ion ratios and limits of acceptance

Compound (3-nitroaniline derivative of analyte)	Transitions	MRM ratio[a]	Acceptance limit, %[b]
2-Fluoro-3′-nitroacetanilide	196.9 → 146.9/196.9 → 122.0	1.01	±20
	196.9 → 117.8/196.9 → 122.0	0.75	±20

[a] Representative MRM ratio. These values are indicative and should be measured for each individual batch.

[b] *See* reference 2.

(**h**) *Control charts*.—Control charts are to be maintained for the method by plotting the relative recovery of the matrix standard quantified from the slope of the recovery curve.

References: (1) New Zealand (Maximum Residue Limits of Agricultural Compounds) Food Standards 2015 (February 20, 2015) *NZ Gazette* **18**

(2) *Off. J. Eur. Commun*. **L221**, 8(2002)

J. AOAC Int. **98**, 1121(2015)
DOI: 10.5740/jaoac.int.2015.02

AOAC SMPR 2015.001
J. AOAC Int. **98**, 1092(2015)
DOI: 10.5740/jaoac.int.SMPR2015.001

Posted: October 16, 2015

OFFICIAL METHODS

Determination of Sodium Monofluoroacetate in Dairy Powders by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS): First Action 2015.02

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Submitted for publication April 2015.
Adopted as a First Action Official Method by the Expert Review Panel on Sodium Monofluoroacetate and approved by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN).
Approved on: March 17, 2015.
DOI: 10.5740/jaoac.int.2015.02

(The method has been validated in cow, goat, and sheep dairy powders, and dairy powder formulations. It can be used for other similar matrixes, including liquid milk, provided that it is demonstrated that the method performance values are met.)

Specific hazards.—*Hydrochloric acid*.—Wear personal protective equipment (PPE) and handle in a fume cupboard.

Sulfuric acid.—Wear PPE and handle in a fume cupboard.

Sodium fluoroacetate.—Wear PPE, including safety glasses and a dust mask, when weighing out the primary material.

Potassium hydroxide.—Wear PPE and handle in a fume cupboard; avoid inhalation of dust.

3-Nitroaniline.—Wear PPE, including safety glasses; avoid inhalation.

Phosphoric acid.—Wear PPE and handle in a fume cupboard.

Sodium fluoroacetate (Figure **2015.02A**) is a rodenticide used in New Zealand to control rats, possums, and rabbits. It is commonly known by its original registration number as compound 1080, but may also be known as sodium monofluoroacetate and fluoroacetic acid sodium salt.

A. Principle

Samples are dissolved in water and extracted into acetone to allow precipitation of proteins. After centrifugation, the solutions are passed through an anion exchange column and eluted with acid to give free fluoroacetic acid. This acid is converted to 2-fluoro-3'-nitroacetanilide via a carbodiimide-mediated amide coupling reaction. The derivative is then subjected to SPE cleanup, eluting with *t*-butyl methyl ether (TBME)–*n*-hexane, concentrated and quantified by LC-MS/MS using derivatized isotopically substituted sodium fluoroacetate as an internal standard. The method reports the analyte as fluoroacetic acid.

B. Apparatus

Note: Where specific equipment is listed, other brands or models may be used provided that they have equivalent performance.

Laboratory equipment.—

(**a**) *Air displacement pipets*.—5000 µL, with long tips.

(**b**) *Autosampler vials*.—2 mL with tapered glass inserts.

(**c**) *Balance*.—2 or 3 decimal top pan.

(**d**) *Balance*.—5 decimal place analytical.

(**e**) *Centrifuge*.—Capable of centrifuging 15 and 50 mL tubes at 4200 × *g* RCF.

(**f**) *Centrifuge tubes*.—15 and 50 mL, tapered polypropylene.

(**g**) *Extraction cartridges*.—Oasis HLB 60 mg.

(**h**) *Glass reservoirs*.— Ground glass, 19/26 joint, approximately 20 mL.

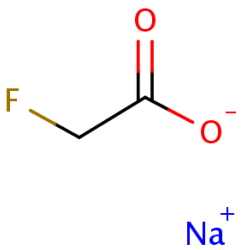


Figure 2015.02A. Sodium fluoroacetate.

(**i**) *Laboratory glassware*.—Measuring cylinders, volumetric flasks, and beakers.

(**j**) *Multi-step dispenser with appropriate tips*.—Eppendorf Stream or equivalent.

(**k**) *Nitrogen blow-down*.—Capable of maintaining a temperature 40 ± 10°C.

(**l**) *pH meter*.

(**m**) *Polypropylene SPE reservoirs*.—10 mL, with adapters.

(**n**) *Positive displacement pipets*.—25, 100, 250, and 1000 µL, with tips.

(**o**) *Refrigerator and freezer.*

(**p**) *Resin chromatography column*.—10 mL polypropylene (Bio-Rad).

(**q**) *Shaker*.—Reciprocating bench top.

(**r**) *Ultrasonic bath*.

(**s**) *Vacuum manifold for SPE cartridges*.—With stopcocks.

(**t**) *Vortex mixer*.

(**u**) *Water bath*.—Maintained at 40 ± 2°C.

Analytical instrumentation.—

(**v**) *LC-MS/MS instrument*.—ABSciex 5500 QTRAP coupled with Agilent 1290 Series HPLC.

(**w**) *HPLC guard column*.—Phenomenex Security C18, 4 × 2 mm.

(**x**) *HPLC column*.—Agilent XDB-C18 100 × 4.6 mm, 1.8 µm.

C. Reagents

All reagents and chemicals must be of such a grade that they do not interfere with the analytical process.

Chemicals.—

(**a**) *Acetone*.—Pesticide grade.

(**b**) *Acetonitrile*.—Pesticide grade.

(**c**) *AG 1-X8 resin*.—100–200 mesh chloride form, ACS reagent grade.

(**d**) *Ammonium acetate*.—ACS reagent grade.

(**e**) *Deionized water*.—Laboratory purified, ≥18 ΩM.

Table 2015.02A. Fortification of recovery samples

Tube No.	Name	Volume, µL WS3	Volume, µL WS2	Volume, µL WS3	Concn fluoroacetic acid, µg/kg
1[a]	Matrix standard	0[a]	0[a]	0[a]	5[a]
2	Recovery 1	0	0	40	0
3	Recovery 2	25	0	40	0.1
4	Recovery 3	125	0	40	0.5
5	Recovery 4	0	25	40	1
6	Recovery 5	0	125	40	5
7	Reagent blank	0	0	40	0

[a] The matrix standard is fortified at step **F**(**b**)(*9*).

(**f**) *Hydrochloric acid*.—Concentrated, ACS reagent grade; 37–38%.

(**g**) *Methanol*.—Pesticide grade.

(**h**) *n-Hexane*.—Pesticide grade.

(**i**) *Phosphoric acid*.—Concentrated, ACS reagent grade.

(**j**) *Potassium dihydrogen phosphate*.—ACS reagent grade.

(**k**) *Potassium hydroxide*.—ACS reagent grade.

(**l**) *Sodium sulfate, anhydrous*.—ACS reagent grade.

(**m**) *Sodium hydrogen carbonate*.—ACS reagent grade.

(**n**) *Sulfuric acid*.—Concentrated, ACS reagent grade.

(**o**) *TBME*.—Pesticide grade.

(**p**) *1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC)*.—ACS reagent grade.

(**q**) *3-Nitroaniline*.—ACS reagent grade.

Solutions.—

(**a**) *5 M hydrochloric acid (2000 mL)*.—(*Caution*: Preparation of solution should be performed inside a fume cupboard.) Into a 2000 mL volumetric flask add 800 mL deionized water. To this add 832 mL of 37–38% (12 M) concentrated hydrochloric acid. Mix and allow to cool. Bring to volume with deionized water. Store at room temperature.

(**b**) *0.2 M hydrochloric acid (2000 mL)*.—Into a 2000 mL volumetric flask add 800 mL deionized water. To this add 80 mL of 5 M hydrochloric acid. Mix and allow to cool, and bring to volume with deionized water. Store at room temperature.

(**c**) *2 M potassium hydroxide (2000 mL)*.—(*Caution*: Preparation of solution should be performed inside a fume cupboard.) Place 1600 mL deionized water in a 2000 mL beaker and place on a magnetic stirrer with follower. Weigh 224.4 g potassium hydroxide into a 500 mL beaker. Add the potassium hydroxide, a few pellets at a time, to the stirred solution. Do not allow the temperature to rise above warm to the touch. When all

Table 2015.02B. Identification parameters for compounds analyzed as negative ions

Compound (3-nitroaniline derivatives of analyte and internal standard)	Expected retention time, min	Molecular ion (Q1)	Product ion (Q3)	Dwell, ms	DP, V	CE, eV	CXP, V
2-Fluoro-3′-nitroacetanilide	2.06	196.931	122.000	50	–120	–24	–17
		196.931	146.900	50	–120	–22	–23
		196.931	117.800	50	–120	–28	–17
1,2-^{13}C-2,2-D-2-fluoro-3′-nitroacetanilide	2.06	201.001	134.900	50	–115	–30	–21
		201.001	45.900	50	–115	–22	–23

the pellets have dissolved, allow the solution to cool and then transfer quantitatively through a glass funnel into a 2000 mL measuring cylinder and bring to volume with deionized water. Store at room temperature.

(**d**) *20 mg/mL 3-nitroaniline (100 mL)*.—(*Caution*: Wear gloves when working with this chemical.) Weigh 2.0 g aliquots of 3-nitroaniline into 100 mL Schott bottles and cap tightly. Store at room temperature.

To one preweighed bottle of 3-nitroaniline add 100 mL acetonitrile using a graduated measuring cylinder. This is sufficient for two batches of 36 sample tubes. Prepare fresh daily.

(**e**) *100 mg/mL EDAC (25 mL)*.—(*Caution*: Exposure to moisture degrades this reagent.) Preweigh 2.5 g aliquots of EDAC into 50 mL polypropylene tubes and cap tightly. Store in a freezer at or below –10°C in a desiccated container.

To one preweighed tube of EDAC add 25 mL deionized water. Prepare fresh daily.

(**f**) *TBME–n-hexane (70 + 30, v/v; 2000 mL)*.—Measure 1400 mL TBME into a 2000 mL Schott bottle and add 600 mL *n*-hexane. Cap and mix. Store at room temperature.

(**g**) *Sulfuric acid in water (25%, v/v; 2000 mL)*.—(*Caution*: Preparation of solution should be performed inside a fume cupboard.) Add approximately 1200 mL deionized water to a 2 L volumetric flask followed by slow addition of 500 mL sulfuric acid. Mix and allow to cool to room temperature. Bring to 2 L volume and store in a Schott bottle. Store at room temperature.

(**h**) *0.05 M potassium dihydrogen phosphate, pH 2.3 (1000 mL)*.—Weigh 6.80 g potassium dihydrogen phosphate into a 500 mL beaker. Add 300 mL deionized water to dissolve the potassium dihydrogen phosphate and quantitatively transfer into a 1 L Schott bottle. Add a further 300 mL deionized water to the beaker to dissolve any remaining potassium dihydrogen phosphate, quantitatively transfer into the Schott bottle, make up to 1 L. Cap, mix, and adjust the pH to 2.3 ± 0.1 with concentrated phosphoric acid. Store at room temperature.

(**i**) *0.1 M sodium hydrogen carbonate (1000 mL)*.—Weigh 8.40 g sodium hydrogen carbonate into a 500 mL beaker. Add 300 mL deionized water to dissolve the sodium hydrogen carbonate and quantitatively transfer into a 1 L Schott bottle. Add a further 300 mL deionized water to the beaker to dissolve any remaining sodium hydrogen carbonate, quantitatively transfer into the Schott bottle, make up to 1 L, cap, and mix to ensure full solubility. Store at room temperature.

(**j**) *AG 1-X8 anion exchange resin*.—Before use, soak the AG 1-X8 anion exchange resin in deionized water for 18–24 h, and store in deionized water until use. Store in a refrigerator.

(**k**) *HPLC mobile phase A: 10 mM ammonium acetate in water (1000 mL)*.—Weigh 0.77 g ammonium acetate into a 1 L Schott bottle, followed by 1000 mL deionized water. Cap and mix to ensure full solubility. Store at room temperature.

(**l**) *HPLC mobile phase B: 10 mM ammonium acetate in 97% acetonitrile (1000 mL)*.—Weigh 0.77 g ammonium acetate into a 50 mL beaker. Use 30 mL deionized water to quantitatively transfer to a 1 L Schott bottle. Stir to fully dissolve and add 970 mL acetonitrile. Sonicate for 10 min to ensure full solubility. Store at room temperature.

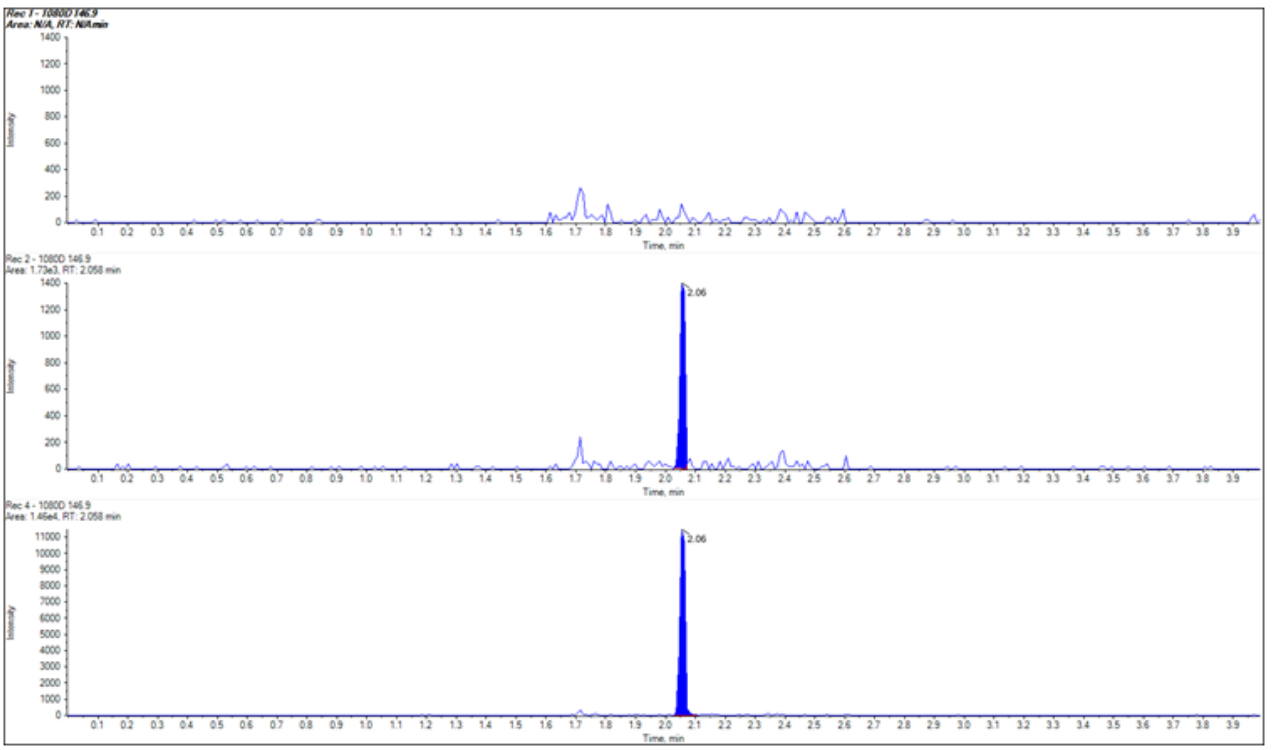


Figure 2015.02B. Chromatograms of confirmation ion for recovery 1 (matrix blank; top), recovery 2 (0.1 µg/kg, ≈LOQ; middle), and recovery 4 (1.0 µg/kg, LOR; bottom).

Table 2015.02C. HPLC solvent gradient

Time	% A (10 mM NH_4Ac in H_2O)	% B (10 mM NH_4Ac in 97% ACN)
0	80	20
2.50	0	100
3.00	0	100
3.01	80	20
4.00	80	20

D. Standards

(**a**) *Primary standards*.—Primary standards are stored in a refrigerator between 2–8°C in the dark. Subsequent solutions should be corrected for purity, moisture, and salt (if applicable). Wear appropriate PPE when weighing out the primary material.

(*1*) *Analytes*.—Sodium fluoroacetate (CAS No. 62-74-8).

(*2*) *Internal standard*.—$^{13}C_2D_2$ sodium fluoroacetate.

(**b**) *Secondary standards*.—(*1*) *Analytes*.—*Fluoroacetic acid (1000 mg/L)*.—Weigh approximately 12.9 mg sodium fluoroacetate into a calibrated 10 mL volumetric flask. Add deionized water, make to volume, and mix until solid is completely dissolved. Transfer to a 15 mL polypropylene screw cap test tube and cap tightly. Store in a freezer at less than –10°C. Calculate exact concentration correcting for purity, moisture (if applicable), and salt using Equation 1.

$$C = [(m \times P \times Mo)/V] \times [MW/MW_{(salt)}] \quad (1)$$

where C = concentration of standard solution in mg/L; m = exact mass of primary standard weighed in mg; P = purity of standard expressed as a decimal equal to % purity/100; Mo = additional purity correction for moisture/water (if applicable). Expressed as a decimal equal to (100 – % moisture)/100; V = volume of solution in L; MW = molecular weight of target analyte; $MW_{(salt)}$ = molecular weight of analyte as salt.

(*2*) *Internal standard*.—*$^{13}C_2D_2$ fluoroacetic acid (500 mg/L)*.—Weigh approximately 7.2 mg (corrected for chemical and isotopic purity) of $^{13}C_2D_2$ labeled sodium fluoroacetate into a calibrated 10 mL volumetric flask. Add deionized water, make to volume, and mix until solid is completely dissolved. Transfer to a 15 mL polypropylene screw cap test tube and cap tightly. Store in the freezer at –10°C or below. Calculate exact concentration correcting for purity, moisture (if applicable), and salt using Equation 1.

(**c**) *Intermediate standards*.—(*1*) *Analyte*.—*Fluoroacetatic acid intermediate standard (50 mg/L)*.—Dilute 2.5 mL (calculate exact volume based on concentration of secondary standard using Equation 2) of fluoroacetic acid secondary standard (1000 mg/L) to 50 mL with deionized water in a calibrated volumetric flask. Use a calibrated positive displacement pipet. Dispense aliquots of the standard into 15 mL polypropylene tubes for frozen storage. Store in the freezer at –10°C or below.

$$C_2 = (C_1 \times V_1)/V_2 \quad (2)$$

where C_2 = concentration of required diluted solution in mg/L; C_1 = concentration of high standard in mg/L; V_1 = volume of high standard required in mL; V_2 = total volume of diluted solution in mL.

(*2*) *Internal standard*.—*$^{13}C_2D_2$ fluoroacetic acid standard (50 mg/L)*.—Dilute 2.5 mL (calculate exact volume based on concentration of secondary standard using Equation 2) of $^{13}C_2D_2$ fluoroacetic acid secondary standard (500 mg/L) to 25 mL with water in a calibrated volumetric flask. Use a calibrated positive displacement pipet. Dispense aliquots of the standard into 15 mL polypropylene tubes. Store in the freezer at –10°C or below.

(**d**) *Working solutions (WS)*.—(*1*) *Analyte*.—(*a*) *WS1 (1.0 mg/L)*.—Dilute 1000 µL fluoroacetic acid intermediate standard (50 mg/L) to 50 mL with deionized water in a calibrated volumetric flask. Dispense aliquots of the WS1 standard into 15 mL polypropylene tubes. Store in the freezer at –10°C or below. A thawed and opened tube WS1 can be stored for up to 2 months in a refrigerator between 2–8°C provided it is resealed and immediately refrigerated after each use.

(*b*) *WS2 (0.1 mg/L)*.—Dilute 10 mL of WS1 (1.0 mg/L) to 100 mL with deionized water in a calibrated volumetric flask. Dispense aliquots of the WS2 standard into 15 mL polypropylene tubes. Store in the freezer at –10°C or below. A thawed and opened tube WS2 can be stored for up to 2 months in a refrigerator between 2–8°C provided it is resealed and immediately refrigerated after each use.

(*c*) *WS3 (0.01 mg/L)*.—Dilute 500 µL of WS2 (0.1 mg/L) to 5 mL with deionized water in a calibrated volumetric flask. Transfer into a 15 mL polypropylene tube. Make fresh daily.

(*2*) *Internal standard*.—*Internal standard working solution (ISWS; 0.5 mg/L)*.—Dilute 1000 µL of $^{13}C_2D_2$ fluoroacetic acid intermediate standard (50 mg/L) to 100 mL with deionized water in a calibrated volumetric flask. Pipet aliquots of the ISWS into 15 mL polypropylene tubes. Store in the freezer at –10°C or below. A thawed and opened tube of ISWS can be stored for up to 2 months in a refrigerator between 2–8°C provided it is resealed and immediately refrigerated after each use.

Table 2015.02D. Instrument parameters for AB Sciex LC-MS/MS system

Parameter	Value
HPLC column	Agilent XDB-C18, 100 × 4.6 mm × 1.8 µm
Column temperature	60°C
Autosampler temperature	10°C
Flow rate	1 mL/min
Injection volume	5 µL
Run time	4 min
Ionization mode	Electrospray
Polarity	Negative
Curtain gas (CUR)	30 psi
Source temp. (TEM)	750°C
Ion source gas 1 (GS1)	60 psi
Ion source gas 2 (GS2)	60 psi
Ion spray voltage (IS)	–4500 V
Collision gas (CAD)	Medium (8)
Entrance potential (EP)	–10 V

Table 2015.02E. Performance values of analytes[a]

Compound	LOD, µg/kg	LOQ, µg/kg	LOR, µg/kg	Within-day CV	Between-day CV (WLR)	U (for 95% CI)	Recovery, % (SD)
Fluoroacetic acid	0.028	0.085	1.0[b]	8.8	9.1	18	97[c] (8.8)
$^{13}C_2$ D_2 Fluoroacetic acid	NA	NA	NA	NA	NA	NA	70[d] (12)

[a] LOD = Limit of detection; LOQ = limit of quantification; CV = coefficient of variation; WLR = within-laboratory reproducibility; U = uncertainty of measurement with a 95% confidence interval; SD = standard deviation.

[b] Limit of reporting (LOR) set according to New Zealand maximum permitted residue limits. *See* reference 1.

[c] Relative recovery.

[d] Absolute recovery.

E. Sampling and Sample Preparation

Preparation of test portion.—Accurately weigh 2.5 ± 0.03 g of room temperature sample into a labeled 50 mL polypropylene tube. In addition to the analytical samples, there are four recovery samples per batch, a reagent blank, and an extra blank for the matrix standard.

F. Procedure

(**a**) *Fortification*.—(*1*) *Analyte*.—Fortify the recoveries as shown in Table **2015.02A** using the working solutions prepared in **D**(**d**). *Note*: Do not add any WS to the matrix blank to be used for the matrix standard.

(*2*) *Internal standard*.—Add 40 µL ISWS to all unknown and recovery samples. *Note*: Do not add any ISWS to the matrix blank to be used for the matrix standard.

(*3*) Allow test portions to equilibrate for 10 min at room temperature.

(**b**) *Extraction*.—(*1*) Place the resin chromatography columns onto a vacuum manifold and fill with 1.4 (±0.2) mL resin. Add 2.5 mL deionized water above the resin bed and close stopcock. Fit suitable reservoirs above the columns.

(*2*) To each test portion add 5 mL water and briefly shake vigorously by hand, cap, and then shake tubes at medium speed on a reciprocating shaker for 5 min to dissolve. Variation to this procedure may be required for atypical matrixes.

(*3*) Add 10 mL acetone to each tube and briefly shake vigorously by hand followed by 2 min on a reciprocating shaker at medium speed.

(*4*) Centrifuge at 4200 × *g* RCF for 10 min.

(*5*) Carefully pour the top solvent layer into the reservoirs above the resin, taking care not to transfer any precipitate.

(*6*) Allow samples to pass through the resin columns under gravity or gentle vacuum, if required.

(*7*) After samples have passed through the resin columns, remove the reservoirs and wash the resin columns with 1 mL of 0.2 M hydrochloric acid. Close stopcock. Do not allow the resin to dry.

(*8*) Place 15 mL polypropylene tubes beneath each resin column. Elute samples with one 5 mL volume of 0.2 M hydrochloric acid at about 30 drops/min. Remove residual hydrochloric acid solution into the collecting tubes under vacuum.

(*9*) To the matrix standard tube only, add 125 µL WS2 and 40 µL ISWS, cap, and vortex mix.

(*10*) To all tubes add 1.25 mL of 20 mg/mL 3-nitroaniline and 0.25 mL of 100 mg/mL EDAC solution followed by 0.5 mL of 2 M potassium hydroxide and 1 mL of 0.05 M potassium dihydrogen phosphate buffer. Cap and mix.

(*11*) Place tubes in a 40 ± 2°C water bath for 20 min.

(*12*) Remove tubes and cool to room temperature.

(*13*) Set up a vacuum manifold with Oasis HLB, 60 mg, 3 mL cartridges.

(*14*) Condition the cartridge with 1 mL methanol. Close the stopcock when the methanol reaches the top frit.

(*15*) Load a portion of the derivatized extract onto the conditioned SPE cartridge.

(*16*) Place an adapter and 10 mL reservoir on top of the cartridge.

(*17*) Transfer the remaining derivatized extract into the reservoir and open the stopcock. Allow to drip slowly to waste at about 30–40 drops/min.

(*18*) When the extract has passed through the cartridge, remove the adapter and reservoir.

(*19*) Wash the cartridge with 2 mL 25% (v/v) sulfuric acid, 1 mL deionized water, 1 mL 0.1 M sodium hydrogen carbonate, and a further 2 mL deionized water to waste.

(*20*) Dry cartridge by applying full vacuum for 5 min.

(*21*) Place 15 mL polypropylene tubes beneath each SPE. Elute the derivatized extract with 2 × 2.5 mL TBME–*n*-hexane (70 + 30, v/v) into the tubes.

(*22*) Dry the cartridge by briefly applying a full vacuum.

(*23*) Check tubes for remaining water. There should be minimal water present. Presence of more than about 50 µL water would indicate inadequate vacuum.

(*24*) Add approximately 200 mg sodium sulfate, anhydrous, to each tube and vortex mix.

(*25*) Centrifuge at 2400 × *g* RCF for 1 min.

Table 2015.02F. Relative retention time (RRT) and limits of acceptance

Compound (3-nitroaniline derivative of analyte)	Monitored compounds	RRT	Acceptance limit[a]
2-Fluoro-3′-nitroacetanilide	Analyte/internal standard	1.004[b]	RRT ± 2.5%

[a] *See* reference 2.

[b] Representative relative retention time. These values are indicative and should be measured for each individual batch.

Table 2015.02G. Ion ratios and limits of acceptance

Compound (3-nitroaniline derivative of analyte)	Transitions	MRM ratio[a]	Acceptance limit, %[b]
2-Fluoro-3′-nitroacetanilide	196.9 → 146.9/196.9 → 122.0	1.01	±20
	196.9 → 117.8/196.9 → 122.0	0.75	±20

[a] Representative MRM ratio. These values are indicative and should be measured for each individual batch.

[b] *See* reference 2.

(*26*) Decant the supernatant into a clean 15 mL tapered, polypropylene tube.

(*27*) Evaporate the solvent to incipient dryness under nitrogen at 40 ± 10°C.

Note: Do not leave on heating block as excess heating may degrade derivatized analyte.

(*28*) Allow tubes to return to near room temperature and then redissolve residue in 150 µL acetonitrile.

(*29*) Vortex mix at low speed.

(*30*) Centrifuge at 2400 × *g* RCF for 1 min.

(*31*) Transfer clear solvent layer to a tapered insert in an autosampler vial, making sure not to transfer any solid and/or particulate matter. Cap firmly.

Note: Final extracts have been shown to be stable at least 5 days when stored in the freezer at –10°C or below.

(**c**) *Instrumental determination*.—(*1*) *Identification parameters*.—Identification parameters for the analysis of sodium fluoroacetic acid are given in Table **2015.02B**.

(*2*) *Analytical instrumentation*.—(*a*) *General*.—Agilent 1290 HPLC system coupled with a 5500 QTRAP Triple Quad Mass Spectrometer. The system is controlled by ABSciex Analyst software. Peak integration is handled with ABSciex MultiQuant Analysis software.

Note: *See* Figure **2015.02B** for exemplary chromatograms.

(*b*) *LC parameters*.—*See* Table **2015.02C** for HPLC solvent gradient.

(*i*) *Column*.—Agilent XDB-C18 100 × 4.6 mm.

(*ii*) *Guard column*.—Phenomenex Security C18, 4 × 2 mm.

(*c*) *Mass spectrometer parameters*.—*See* Table **2015.02D** for full analytical parameters.

G. Calculations

Quantification of fluoroacetic acid is based on peak area. Matrix recoveries are used to generate calibration curves. An unknown peak that falls within the evaluation window (as calculated by recoveries and internal standard) is quantified from the appropriate calibration curve and the value tabulated, together with peak identification information. Each potential unknown is then manually assessed for the quality of identification by viewing integrated chromatograms and those of any qualifying ions.

$$C_u = RR/Sl$$

where C_u = concentration of unknown sample in µg/kg; *RR* = relative response of unknown sample; *Sl* = slope of calibration curve.

H. Method Performance and Quality Control

(**a**) *Reagent blank test*.—A reagent blank (deionized water) test is performed with each batch.

(**b**) *Matrix standard test*.—Performed with each batch according to Table **2015.02A**.

(**c**) *Matrix blank test (Recovery 1)*.—A matrix blank test is performed with each batch.

(**d**) *Matrix recovery test (recovery samples)*.—Performed with each batch according to Table **2015.02A**.

(**e**) *Certified reference materials (CRM)*.—No CRM is currently available. In practice, external checks of the method are performed by participation in interlaboratory calibration studies when available.

(**f**) *Performance values*.—Values found in Table **2015.02E** are calculated from the in-house single-laboratory validation (SLV)completed by AsureQuality Ltd.

(**g**) *Acceptance criteria*.—(*1*) *Individual sample acceptance criteria*.—The internal standard response for an individual sample should exceed 33% of the mean internal standard response of the recovery samples.

(*2*) *Batch acceptance criteria*.—Analyte relative recoveries for the recovery samples should be within 3 SD of the mean relative recovery established from control charts. Calibration curves should have a coefficient of determination $R^2 > 0.95$.

(*3*) *Positive sample acceptance criteria*.—Retention time acceptance criteria are given in Table **2015.02F**. Ion ratio acceptance limits are given in Table **2015.02G**.

(**h**) *Control charts*.—Control charts are to be maintained for the method by plotting the relative recovery of the matrix standard quantified from the slope of the recovery curve.

References

(1) New Zealand (Maximum Residue Limits of Agricultural Compounds) Food Standards 2015 (February 20, 2015) *NZ Gazette* **18**

(2) *Off. J. Eur. Commun*. **L221**, 8(2002)

(3) AOAC SMPR 2015.001 *J. AOAC Int*. (future issue)

AOAC Official Method 2015.03
Sodium Fluoroacetate in Infant Formula
Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)
First Action 2015

[Applicable for the quantitative determination of sodium fluoroacetate in liquid and powdered milk- and soy-based infant formulas by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The limit of quantification (LOQ) of sodium fluoroacetate is 1 µg/kg by this method. Application of this method to matrices not covered by the scope of application requires an additional validation.]

Caution: Material Safety Data Sheets (MSDS) should be available for all chemicals; inherent risks and corresponding safety precautions shall be identified.

Follow general safety precautions and environmental aspects as described in the local Safety, Health and Environment rules in place.

Sodium fluoroacetate is highly toxic to humans. Take all necessary precautions, especially when working with concentrated stock standard solutions.

Sodium fluoroacetate (Figure **2015.03A**) is a synthetic pesticide known as "1080" and used to fight mammalian pest species. Farmers and graziers use the poison to protect pastures and crops from various herbivorous mammals. It is used as well to protect sheep and goats from predatory coyotes (predacide). In New Zealand and Australia, it is used to control invasive non-native mammals that prey on or compete with native wildlife and vegetation. Sodium fluoroacetate is highly toxic to mammals, including humans. This pesticide is approved for use in the following countries: United States, Canada, Mexico, Australia, New Zealand, Korea, Japan, and Israel. New Zealand has used "1080" for pest control since the 1950s, while the United States began use in the 1940s.

Sodium fluoroacetate is also a naturally occurring poison found in at least 40 plants native in Australia, South and West Africa, and Brazil.

A. Principle

Milk powder is first reconstituted in water. Liquid sample is used as such. Acetonitrile is added to precipitate proteins. After centrifugation, the supernatant is washed with hexane and then acidified with concentrated sulfuric acid. QuEChERS salts ($MgSO_4$ and NaCl) are added for phase separation and the mixture is centrifuged. The resulting supernatant is evaporated to 0.5 mL remaining volume and centrifuged before LC-MS/MS analysis in selected reaction monitoring (SRM) by electrospray ionization (ESI) in negative mode. The compound is analyzed as its fluoroacetate anion.

Quantification is performed by the isotopic dilution approach using ^{13}C labeled sodium fluoroacetate as internal standard (IS).

Positive identification of fluoroacetate in samples is conducted according to the confirmation criteria defined in EU Commission Decision 2002/657/EC (1).

B. Chemicals and Materials

Commercial references are only a guideline. Use equivalent chemicals or materials when listed items are not locally available.

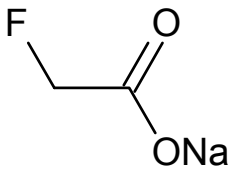


Figure 2015.03A. Chemical structure of sodium fluoroacetate ($NaFC_2H_2O_2$; CAS No. 62-74-8; MW 100 g/mol).

(**a**) *Chemicals*.—Before using chemicals, refer to the Sigma-Aldrich (www.sigmaaldrich.com) Guide to Chemical Safety and/or other adequate manuals or safety data sheets approved by your local authorities and ensure that the safety guidelines are applied.

(*1*) *Water for chromatography*.—CAS No. 7732-18-5 (e.g., Merck LiChrosolv® art. 15333; www.emdmillipore.com).

(*2*) *Acetonitrile, hypergrade for LC-MS*.—CAS No. 75-05-8 (e.g., Merck LiChrosolv art. 100029; www.chemdat.info).

(*3*) *n-Hexane*.—For gas chromatography; CAS No. 110-54-3 (e.g., Merck SupraSolv® art. 104371; www.chemdat.info).

(*4*) *QuEChERS extraction packets*.—10 g, 200 foil packs per box, each pack containing 4 g magnesium sulfate ($MgSO_4$) and 1 g sodium chloride (NaCl). CAS Nos. 7487-88-9 and 7647-14-5 (e.g., Agilent art. 5982-7550; www.agilent.com).

(*5*) *Ammonium formate*.—LC-MS ultra, eluent additive for UHPLC-MS. CAS No. 540-69-2 (e.g., Fluka art. 14266; www.sigmaaldrich.com).

(*6*) *Sulfuric acid*.—Concentrated, w = 95–97%. CAS No. 7664-93-9 (e.g., Merck art. 100731; www.merckmillipore.com).

(*7*) *Formic acid*.—Concentrated (e.g., Merck art. 100264; www.chemdat.info). CAS No. 64-18-6.

(*8*) *Sodium fluoroacetate*.—CAS No. 62-74-8, w = 99%, 10 µg/mL in water (e.g., Dr. Ehrenstorfer art. DRE-L13772000AL; www.lgcstandards.com).

(*9*) *$^{13}C_2$-Sodium fluoroacetate*.—w = 99%, isotopic purity >99.5% (e.g., BDG Synthesis art. 130042-10; http://bdg.co.nz).

(**b**) *Materials*.—

(*1*) *Falcon tubes, conical, polypropylene*.—50 mL (e.g., Becton Dickinson Labware art. 352070; www.bdbiosciences.com).

(*2*) *Falcon tubes, conical, polypropylene*.—15 mL (e.g., Becton Dickinson Labware art. 352097; http://www.bdbiosciences.com).

(*3*) *Centrifuge with rotors adapted for 50 and 15 mL tubes*.—4000 × *g*, temperature controlled (e.g., Multifuge Heraeus; www.thermo.com).

(*4*) *Vortex*.—e.g., Millian Genie 2 (http://www.milian.com).

(*5*) *Centrifuge with rotor adapted for 2 mL tubes*.—17 000 × *g* (e.g., Heraeus Frisco 17; www.thermoscientific.com).

(*6*) *Microcentrifuge tubes, polypropylene*.—2 mL (e.g., Trefflab art. 9607246901; www.treff-ag.ch).

(*7*) *Analytical balance*.—With precision range 0.01 mg.

(*8*) *Shaker*.—GenoGrinder Model 2010 (www.spexsampleprep.com).

(*9*) *Evaporator*.—e.g., Reacti-Vap Evaporator (art. TS-18825; www.thermo.com).

(**c**) *Special equipment and instrumentation*.—Where a specific model is cited, an alternative may be used if it has the same characteristics.

(*1*) *HPLC system*.—Agilent 1200 SL (www.agilent.com) coupled to a Sciex 5500 triple stage quadrupole mass spectrometer

equipped with a TurboIonSpray® ionization source (www.sciex.com).

(*2*) *HPLC column*.—Acquity UPLC BEH Amide, 2.1 × 100 mm, 1.7 µm (Waters art. 186004801; www.waters.com).

(**d**) *Glassware decontamination*.—No specific requirement.

C. Preparation of Reagents

Volumes of glassware are purely indicative and may be modified as long as the proportion of reagents is maintained.

(**a**) *Sodium fluoroacetate stock standard solution, 10 µg/mL in water*.—The stock standard solution is available as ready-to-use 10 mL solution. Store at room temperature for the time given in the certificate of analysis.

(**b**) *Sodium fluoroacetate working standard solution, 1.0 µg/mL in acetonitrile–water (9 + 1)*.—Into a 10 mL volumetric flask, pipet 1.0 mL of the stock standard solution 10 µg/mL, **C**(**a**). Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**c**) *Sodium fluoroacetate working standard solution, 0.2 µg/mL in acetonitrile*.—Into a 10 mL volumetric flask, pipet 2.0 mL of the stock standard solution 1 µg/mL, **C**(**b**). Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**d**) *Sodium fluoroacetate working standard solution, 0.05 µg/mL in acetonitrile*.—Into a 10 mL volumetric flask, pipet 2.5 mL of the stock standard solution 0.2 µg/mL, **C**(**c**). Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**e**) *$^{13}C_2$-Sodium fluoroacetate (IS) stock standard solution, 1000 µg/mL in water*.—Into a 10 mL volumetric flask, weigh 10 ± 0.1 mg of standard. Dissolve and complete to the mark with water.

Alternatively (to minimize analyst exposure during weighing) weigh the container containing the analyte first (w_1, in mg), then transfer its whole content into a 10 mL volumetric flask. Dissolve and complete to mark with water for chromatography. Weigh again the empty original container once dried (w_2, in mg). Concentration of this solution in µg/mL is $1000 \times (w_1 - w_2)/10$. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**f**) *$^{13}C_2$-Sodium fluoroacetate (IS) working standard solution, 10 µg/mL in acetonitrile*.—Into a 10 mL volumetric flask, pipet 100 µL of the stock solution 1000 µg/mL, **C**(**e**). Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**g**) *$^{13}C_2$-Sodium fluoroacetate (IS) working standard solution, 0.2 µg/mL in acetonitrile*.—Into a 50 mL volumetric flask, pipet 1000 µL of the working standard solution 10 µg/mL, **C**(**f**). Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**h**) *Standard solutions for calibration curve*.—Into six separate 5 mL volumetric flasks, transfer the volumes of working standard solutions as described in Table **2015.03A**. Complete to the mark with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**i**) *Solutions for LC-MS/MS*.—

(*1*) *Mobile phase A, water containing 5 mM ammonium formate and 0.01% (v/v) formic acid*.—Into a weighing boat, weigh 315 ± 5 mg ammonium formate. Transfer this mass into a 1000 mL volumetric flask. Add approximately 300 mL water for chromatography and mix to dissolve. Add 100 µL concentrated formic acid. Complete to volume with water for chromatography. Mix. Store at room temperature for no longer than 1 month.

Table 2015.03A. Pipetting schema for the calibration curve

	Standard					
	1	2	3	4	5	6
Working standard solution of sodium fluoroacetate, 0.2 µg/mL, **C**(**c**), µL	0	50	150	300	500	1000
Working standard solution of IS, 0.2 µg/mL, **C**(**g**), µL	500	500	500	500	500	500
Acetonitrile	Complete to the 5 mL mark					
This corresponds to:						
Concentration of sodium fluoroacetate, ng/mL	0	2	6	12	20	40
Concentration of IS, ng/mL	20	20	20	20	20	20

(*2*) *Mobile phase B, acetonitrile*.—Use acetonitrile hyper grade for LC-MS.

(*3*) *Solution for flushing injection port, acetonitrile–water (1 + 1)*.—Into a 1000 mL volumetric flask, transfer by means of graduated cylinder, 500 mL of acetonitrile gradient grade for chromatography. Complete to volume with water for chromatography. Transfer into an HPLC bottle. Store at room temperature for no longer than 1 month.

D. Sampling and Preparation of Test Samples

(**a**) *Sampling procedure*.—A representative sample (minimum 100 g or 100 mL) should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

(**b**) *Laboratory sample*.—Store in the laboratory at room temperature until analysis, unless otherwise mentioned.

(**c**) *Test sample preparation*.—

(*1*) *Powdered sample*.—Mix well the powdered laboratory sample by means of a spoon before taking a test portion. Alternatively, transfer the whole sample into a container of capacity about twice that of the laboratory sample volume. Close the container immediately. Mix thoroughly by repeatedly shaking and inverting the container.

(*2*) *Liquid sample*.—Shake thoroughly the container containing the sample.

E. Preparation of Test Portions and Extraction Procedure

QC samples (certified, *P*-test, in-house reference samples, or spiked samples) must be regularly included and analyzed in duplicate. Different product types should be analyzed regularly in duplicate.

If necessary, different sized glassware may be substituted for specific volumes listed during the preparation of test solutions as long as the proper dilutions ratios are maintained.

(**a**) *Test portion preparation*.—

(*1*) *Powdered sample*.—Into a 50 mL polypropylene Falcon tube, weigh 5.0 ± 0.1 g powdered sample, **D**(**c**). Record the mass to 0.1 g.

Add 20 mL water for chromatography. Mix thoroughly by inversion and place onto a GenoGrinder shaker. Shake for 1.5 min at 1500 rpm. No lump should be visible.

Transfer 5.0 ± 0.1 g of this slurry into a 15 mL polypropylene Falcon tube. Record the mass to 0.1 g.

Add 50 µL of the IS working solution 0.2 µg/mL, **C**(**g**). Mix thoroughly and make sure that the spiked volume is totally absorbed by the matrix. This spike corresponds to 10 µg/kg equivalent-in-sample concentration of IS.

(*2*) *Liquid sample*.—Into a 15 mL polypropylene Falcon tube, weigh 5.0 ± 0.1 g of liquid sample, **D**(**c**).

Add 250 µL of the IS working solution 0.2 µg/mL, **C**(**g**). Mix thoroughly and make sure that the spiked volume is totally absorbed by the matrix. This spike corresponds to 10 µg/kg equivalent-in-sample concentration of IS.

(**b**) *Extraction procedure*.—To the test portion prepared as described in **E**(**a**)(*1*) or **E**(**a**)(*2*), add 8 mL acetonitrile. Mix thoroughly. Place onto a GenoGrinder shaker and shake for 1.5 min at 1500 rpm.

Centrifuge at 4000 × *g* at room temperature for 5 min and transfer the supernatant (approximately 9 to 10 mL) into a 50 mL Falcon tube.

Add 10 mL hexane. Place onto a GenoGrinder shaker and shake for 1.5 min at 1500 rpm.

Centrifuge at 4000 × *g* at room temperature for 5 min. Pipet the upper hexane phase and discard it to waste.

Add 100 µL of concentrated sulfuric acid (H_2SO_4) to the solution containing the analyte. Mix thoroughly. The resulting pH must be ≤1 to have the analyte in its acidic form (pKa of fluoroacetic acid is 2.39).

Add a buffer salt mixture (Agilent QuEChERS ready-to-use mix) containing 4.0 ± 0.4 g $MgSO_4$ and 1.0 ± 0.1 g NaCl. Immediately hand-shake by inversion or by vortexing to prevent any lump formation. Place onto a GenoGrinder shaker and shake for 1.5 min at 1500 rpm.

Centrifuge at 4000 × *g* at room temperature for 5 min and transfer the supernatant (approximately 5 mL) into a 15 mL Falcon tube.

Evaporate the collected supernatant under a stream of nitrogen at 40 ± 2°C until a 0.5 mL remaining volume. A mark at the 0.5 mL level is visible onto the tube. Do not evaporate to lower volumes to prevent loss on evaporation.

Transfer the 0.5 mL remaining volume into a 2 mL tube and centrifuge at 17 000 × *g* at room temperature for 5 min.

Transfer the clear supernatant into an HPLC vial for further LC-MS/MS analysis.

(**c**) *Reagent blank*.—In order to control any contamination during the sample workup, a reagent blank must be analyzed along with each series of routine samples. Water is used instead of milk. Proceeded exactly as described in **E**(**a**) and (**b**).

F. Instrumental Conditions

(**a**) *LC-MS/MS analysis*.—Where a specific instrument is cited, an alternative may be used provided it has the same or better characteristics. As well, an alternative HPLC column may be used provided it allows a retention time of the eluting analyte that is at least twice the retention time corresponding to the void volume of the column.

(*1*) *HPLC conditions*.—Using an Agilent 1200 SL HPLC system (*see* Table **2015.03B**). *See* Table **2015.03C** for LC gradient.

Using these conditions, the compound elutes at approximately 1.7 min (*see* Figures **2015.03B**–**E**).

(*2*) *MS parameters*.—MS parameters (Tables **2015.03D** and **E**) are obtained by separately syringe-infusing standard solution (approximately 1 µg/mL) of each unlabeled and labeled compounds (syringe flow rate of 10 µL/min) along with the HPLC flow at 0.45 mL/min using a T connector. The HPLC flow is constituted with 10% A, **C**(**i**)(*1*), and 90% B, **C**(**i**)(*2*).

Table 2015.03B. HPLC conditions for the analysis of sodium fluoroacetate

Mobile phase A	Water containing 5 mM ammonium formate and 0.01% formic acid, **C**(**i**)(*1*)
Mobile phase B	Acetonitrile, **C**(**i**)(*2*)
Injection volume	20 µL
Column	Waters Acquity UPLC BEH Amide, 2.1 × 100 mm, 1.7 µm
Column oven temp.	45°C
Flow rate	0.45 mL/min
Needle wash	In flush port for 20 s using acetonitrile–water (1 + 1) solution, **C**(**i**)(*3*)
Diverter valve	HPLC flow is directed into the MS detector between 1.0 and 2.5 min
Gradient	LC gradient is described in Table **2015.03C**

(**b**) *Instrument check test*.—Before routine analysis, ensure that the LC-MS/MS apparatus is working in conditions such as the method remains fit for purposes. This involves to inject a low concentration calibrant [e.g., STD 2, **C**(**h**)] to check that sensitivity of the instrument is adequate.

G. Operating Procedure and Determination

(**a**) *Sequence setup*.—Inject solutions in the following order: acetonitrile (as blank solvent) at least three times, standard solutions, **C**(**h**), acetonitrile at least three times, reagent blank, **E**(**c**), extract solutions, **E**(**b**), and standard solutions, **C**(**h**), again. Inject acetonitrile after each three to four extract solutions to check for any carry-over.

(**b**) *Calibration*.—Draw a calibration curve by plotting peak area ratio of the analyte and its IS (= *y* axis) against concentration ratio of the analyte and its IS (= *x* axis). Calculate the slope and intercept by linear regression. Check the linearity of the calibration [regression coefficient R^2 should be higher than 0.98 and relative standard deviation of the average of response factors (= *y*/*x*) should be <15%].

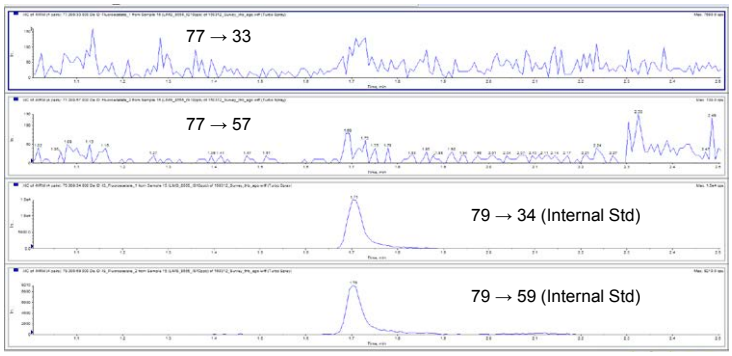
(**c**) *Identification and confirmation*.—Sodium fluoroacetate is identified and confirmed when the following criteria are fulfilled (1).

(*1*) The ratio of the chromatographic retention time of the analyte to that of its IS, i.e., the relative retention time, corresponds to that of the averaged relative retention time of the calibration solutions within a ±2.5% tolerance.

Table 2015.03C. LC gradient used for analysis of sodium fluoroacetate

Time, min	A, %	B, %
0	10	90
2.0	10	90
3.0	60	40
4.5	60	40
4.6	10	90
8.0	10	90

Milk Infant Formula
Lactose Free
LIMS 890000008555
Unspiked



Milk Infant Formula
Lactose Free
LIMS 890000008555
Spiked at 1 µg/kg

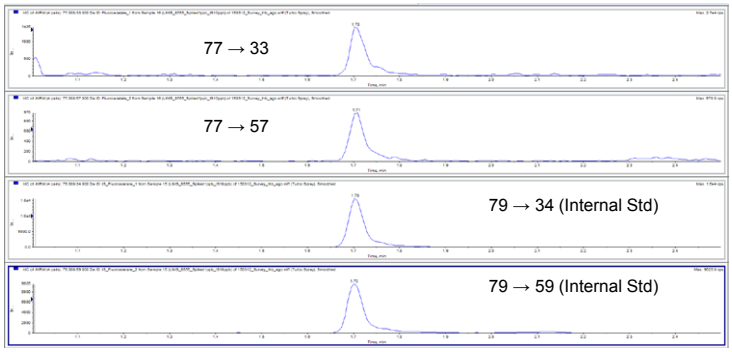
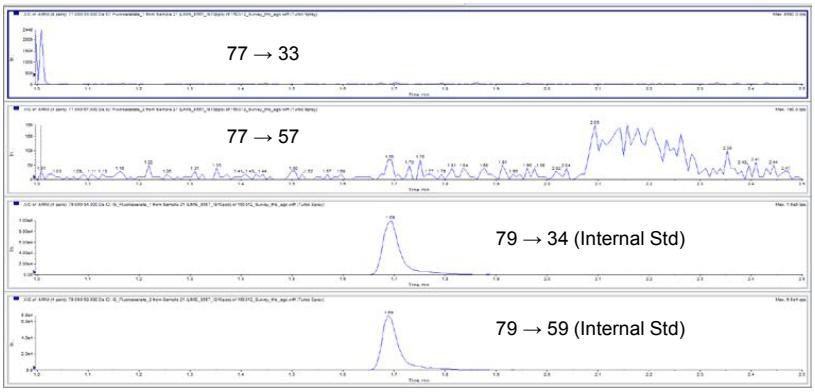


Figure 2015.03B. LC-MS/MS chromatograms of a powdered milk-based infant formula (lactose free) unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Milk Infant Formula
Ready-to-feed (liquid)
LIMS 890000008557
Unspiked



Milk Infant Formula
Ready-to-feed (liquid)
LIMS 890000008557
Spiked at 1 µg/kg

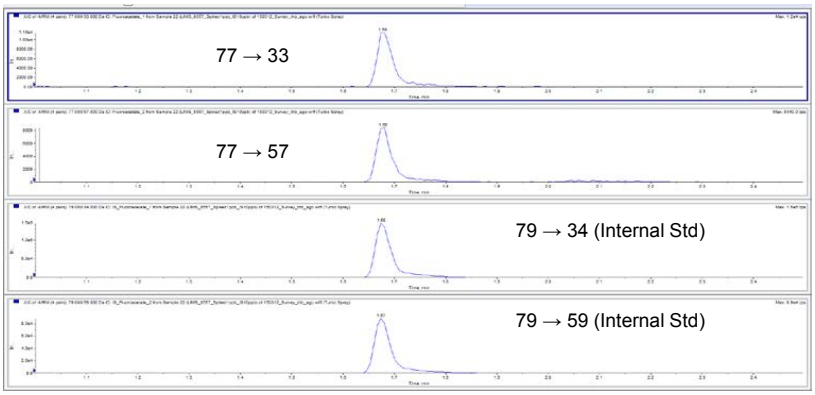
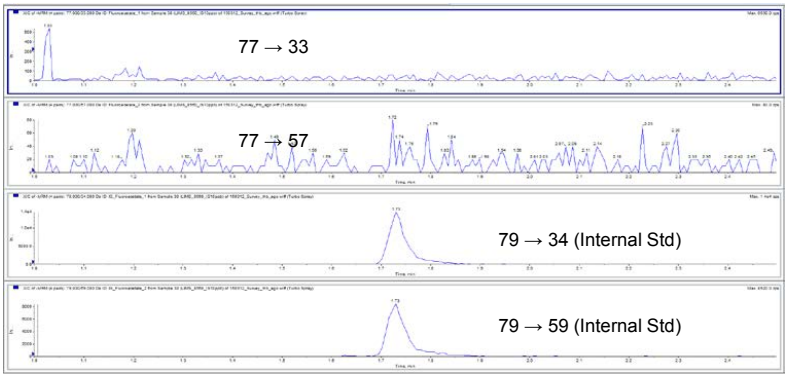


Figure 2015.03C. LC-MS/MS chromatograms of a liquid milk-based infant formula (ready-to-feed) unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Infant Formula
Soya-based
LIMS 890000008560
Unspiked



Infant Formula
Soya-based
LIMS 890000008560
Spiked at 1 µg/kg

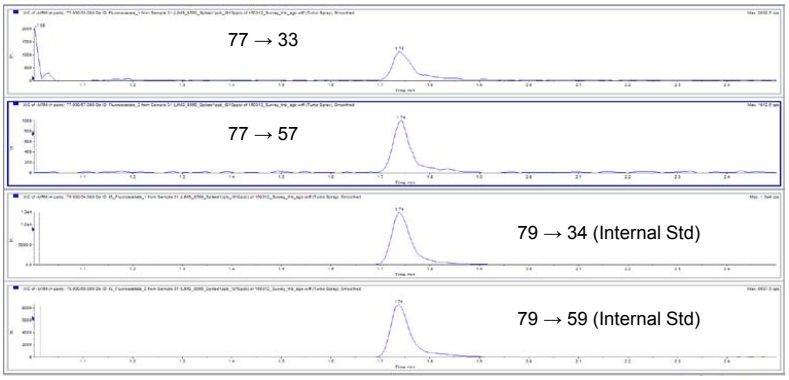
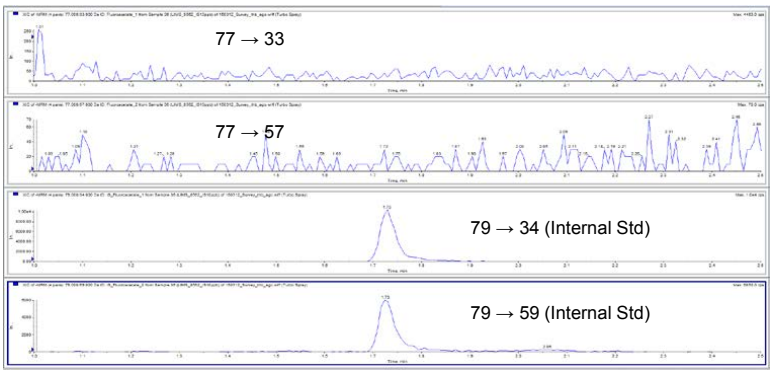


Figure 2015.03D. LC-MS/MS chromatograms of a powdered soya-based infant formula unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Milk Infant Formula
LIMS 890000008562
Unspiked



Milk Infant Formula
LIMS 890000008562
Spiked at 1 µg/kg

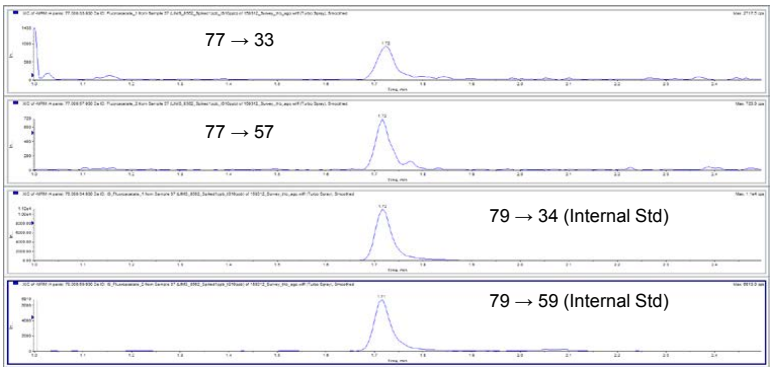


Figure 2015.03E. LC-MS/MS chromatograms of a powdered milk infant formula unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Table 2015.03D. Typical MS parameters for the analysis of sodium fluoroacetate

Parameter	Applied Biosystems Sciex 5500
Ionization type	Electrospray (ESI)
Polarity	Negative ionization
Spray voltage	–4500 V
Source block temperature	500°C
Gas	Curtain gas: 30 psi Ion source gas 1 (GS1): 40 psi Ion source gas 2 (GS2): 40 psi
Source position adjustments	Vertical micrometer value: 5.0 Horizontal micrometer value: 5.0 Electrode protusion: 1.0 mm
Collision energy (CE)	–15
Entrance potential (EP)	–10 V
Collision exit potential (CXP)	–9 V
Declustering potential (DP)	–45 V
CAD gas pressure (MRM)	Medium (6)
Resolution	High on each quadrupole
Scan time (for each transition)	100 ms

(*2*) The peak area ratios from the different transition reactions recorded for the analyte and its IS are within the tolerances fixed by the EU criteria (1) as shown in Table **2015.03E**.

(**d**) *Time of analysis*.—Following this procedure, 20 samples can be analyzed within 24 h.

H. Calculations and Expression of Results

(**a**) *Calculation*.—Calculate the mass fraction, *w*, of sodium fluoroacetate in microgram per kilogram of sample (µg/kg), using the equation:

$$w = \frac{\left(\frac{A_a}{A_s}\right) - I}{S} \times \frac{m_s}{m_a}$$

where A_a = peak area of the analyte in the sample (transition reaction used for quantification); A_{is} = peak area of the IS in the sample (transition reaction used for quantification); I = intercept of the regression line for the transition reaction used for quantification; S = slope of the regression line for the transition reaction used for quantification; m_{is} = mass of IS added to the test portion, in ng (i.e., 10 ng for powdered sample and 50 ng for liquid sample); m_a = mass of the test portion, in g (i.e., 1 g for powdered sample and 5 g for liquid sample).

Table 2015.03F. Samples considered for the validation (SPIFAN kit)

Infant formulae	Batch	Manufacturer (USA)
Milk-based	K16NTAV	PBM Nutritionals
Soy-based	E10NWZC	PBM Nutritionals
Partially hydrolyzed milk-based	410057652Z	Nestlé
Partially hydrolyzed soy-based	410457651Z	Nestlé
High-fat nutritional	00729RF00	Abbott Nutrition
High-protein nutritional	00730RF00	Abbott Nutrition

(**b**) *Expression of results*.—Report the result of sodium fluoroacetate in µg/kg with one significant figure. Nondetected amount must be expressed as <1 µg/kg.

I. Performance Characteristics

The method was validated using samples provided by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). Infant formulae considered are described in Table **2015.03F**.

Validation was performed according to the protocol described in SANCO/12571/2013 (2): precision data were obtained by spiking each sample at 1 and 10 µg/kg concentration levels (respectively corresponding to 1 × LOQ and 10 × LOQ level). At least two operators were involved in these experiments, each performing five replicates at the mentioned fortification levels on two different days (leading to a total of 10 separate experiments for each fortification level). Nonfortified samples were analyzed as well to verify absence of the pesticide before fortification trials.

(**a**) *Linearity*.—Linearity was verified over the 0–2 area ratio range, corresponding to 0–0.8 ng sodium fluoroacetate (0.4 ng IS) injected on-column. The calibration follows a linear model with R^2 > 0.99 and relative standard deviation of the average of response factors <15%.

(**b**) *LOQ*.—LOQ is 1 µg/kg for infant formulae. This concentration corresponds to the lowest fortification level considered during validation.

(**c**) *Recovery, repeatability, and intermediate reproducibility precisions*.—Precision data are described in Table **2015.03G**. All performance data fulfilled SANCO/12571/2013 requirements, i.e., both CV(r) and CV(iR) ≤ 20% and recoveries within the 70–120% range. Exception was recovery for a milk-based formula at the 1 µg/kg fortification level (134%). A small peak was observed at the transition reaction used for quantification in the related unfortified sample extract, meaning that sodium fluoroacetate might be present at a concentration well below the quantification capability of this method. This may explain this higher recovery value.

Table 2015.03E. Transition reactions monitored for the analysis of sodium fluoroacetate (as its fluoroacetate anion) and its corresponding IS and peak area ratios along with their limit of acceptance according to CD 2002/657/EC (1)

	Transition reactions (*m/z*) used for		
	Quantification	Analyte confirmation	Peak area ratio ± limit, %
Fluoroacetate	77.0 → 33.0[a]	77.0 → 57.0[a]	0.80 ± 20
$^{13}C_2$-Fluoroacetate (IS)	79.0 → 34.0	79.0 → 59.0	0.68 ± 20

[a] *m/z* 57 corresponds to the loss of hydrofluoric acid $[M\text{-}HF]^-$ and *m/z* 33 to the loss of carbone dioxide $[M\text{-}CO_2]^-$.

Table 2015.03G. Performance characteristics

	Recovery, %[a]		CV(r), %		CV(iR), %	
Sample	1 µg/kg	10 µg/kg	1 µg/kg	10 µg/kg	1 µg/kg	10 µg/kg
Milk-based formula	134	105	6	3	11	4
Soy-based formula	117	103	6	2	8	3
Partially Hydrolyzed milk-based formula	111	108	4	5	9	4
Partially hydrolyzed soy-based formula	116	103	9	2	8	2
High-fat nutritional formula	112	101	4	3	7	4
High-protein nutritional formula	96	99	4	2	6	2

[a] Recovery data for sodium fluoroacetate were calculated from values obtained under intermediate reproducibility conditions.

J. Internal Control Plan

QC samples (certified, *P*-test, in-house reference samples, or spiked samples) must be regularly included and analyzed in duplicate.

Spiked experiment.—Spike the test portion with 20 µL (for powdered matrices) or 100 µL (for liquid matrices) of standard working solution 0.05 µg/mL, **C**(**d**), at the same time as the IS is added, **E**(**a**). This corresponds to 10 µg/kg spiking level. Calculate the recovery rate (Rec) of the spiked sample using the following equation:

$$\mathrm{Re}\,c = \frac{\rho_T - \rho_N}{\rho_{Spiked}} \times 100$$

where ρ_T is the total concentration of sodium fluoroacetate measured in the spiked sample in micrograms per kg. ρ_N is the native concentration of sodium fluoroacetate measured in the nonspiked sample in micrograms per kg. ρ_{Spiked} is the concentration of sodium fluoroacetate spiked in the sample in micrograms per kg (calculated value).

The recovery rate should be between 70–120% when spiked at the 1 µg/kg level.

References: (1) Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, *Off. J. Eur. Commun.* (2002) **L221**, 8–36. http://eur-lex.europa.eu/legal-content/EN/TXT/F/?uri=CELEX:32002D0657&rid=1

(2) SANCO/12571/2013: Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed, http://ec.europa.eu/food/plant/pesticides/guidance_documents/docs/qualcontrol_en.pdf

J. AOAC Int. **98**, 1127(2015)
DOI: 10.5740/jaoac.int.2015.03

AOAC SMPR 2015.001
J. AOAC Int. **98**, 1092(2015)
DOI: 10.5740/jaoac.int.SMPR2015.001

Posted: October 16, 2015

OFFICIAL METHODS

Determination of Sodium Fluoroacetate in Infant Formula by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS): First Action 2015.03

PASCAL MOTTIER
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Submitted for publication March 2015.
Adopted as a First Action Official Method by the Expert Review Panel on Sodium Fluoroacetate (Compound 1080) and approved by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN).
Approved on: March 16, 2015.
DOI: 10.5740/jaoac.int.2015.03

[Applicable for the quantitative determination of sodium fluoroacetate in liquid and powdered milk- and soy-based infant formulas by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The limit of quantification (LOQ) of sodium fluoroacetate is 1 µg/kg by this method. Application of this method to matrices not covered by the scope of application requires an additional validation.]

Caution: Material Safety Data Sheets (MSDS) should be available for all chemicals; inherent risks and corresponding safety precautions shall be identified.

Follow general safety precautions and environmental aspects as described in the local Safety, Health and Environment rules in place.

Sodium fluoroacetate is highly toxic to humans. Take all necessary precautions, especially when working with concentrated stock standard solutions.

Sodium fluoroacetate (Figure **2015.03A**) is a synthetic pesticide known as "1080" and used to fight mammalian pest species. Farmers and graziers use the poison to protect pastures and crops from various herbivorous mammals. It is used as well to protect sheep and goats from predatory coyotes (predacide). In New Zealand and Australia, it is used to control invasive non-native mammals that prey on or compete with native wildlife and vegetation. Sodium fluoroacetate is highly toxic to mammals, including humans. This pesticide is approved for use in the following countries: United States, Canada, Mexico, Australia, New Zealand, Korea, Japan, and Israel. New Zealand has used "1080" for pest control since the 1950s, while the United States began use in the 1940s.

Sodium fluoroacetate is also a naturally occurring poison found in at least 40 plants native in Australia, South and West Africa, and Brazil.

A. Principle

Milk powder is first reconstituted in water. Liquid sample is used as such. Acetonitrile is added to precipitate proteins. After centrifugation, the supernatant is washed with hexane and then acidified with concentrated sulfuric acid. QuEChERS salts ($MgSO_4$ and NaCl) are added for phase separation and the mixture is centrifuged. The resulting supernatant is evaporated to 0.5 mL remaining volume and centrifuged before LC-MS/MS analysis in selected reaction monitoring (SRM) by electrospray ionization (ESI) in negative mode. The compound is analyzed as its fluoroacetate anion.

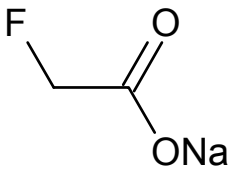


Figure 2015.03A. Chemical structure of sodium fluoroacetate ($NaFC_2H_2O_2$; CAS No. 62-74-8; MW 100 g/mol).

Quantification is performed by the isotopic dilution approach using ^{13}C labeled sodium fluoroacetate as internal standard (IS).

Positive identification of fluoroacetate in samples is conducted according to the confirmation criteria defined in EU Commission Decision 2002/657/EC (1).

B. Chemicals and Materials

Commercial references are only a guideline. Use equivalent chemicals or materials when listed items are not locally available.

(**a**) *Chemicals*.—Before using chemicals, refer to the Sigma-Aldrich (www.sigmaaldrich.com) Guide to Chemical Safety and/or other adequate manuals or safety data sheets approved by your local authorities and ensure that the safety guidelines are applied.

(*1*) *Water for chromatography.*—CAS No. 7732-18-5 (e.g., Merck LiChrosolv® art. 15333; www.emdmillipore.com).

(*2*) *Acetonitrile, hypergrade for LC-MS*.—CAS No. 75-05-8 (e.g., Merck LiChrosolv art. 100029; www.chemdat.info).

(*3*) *n-Hexane*.—For gas chromatography; CAS No. 110-54-3 (e.g., Merck SupraSolv® art. 104371; www.chemdat.info).

(*4*) *QuEChERS extraction packets*.—10 g, 200 foil packs per box, each pack containing 4 g magnesium sulfate ($MgSO_4$) and 1 g sodium chloride (NaCl). CAS Nos. 7487-88-9 and 7647-14-5 (e.g., Agilent art. 5982-7550; www.agilent.com).

(*5*) *Ammonium formate*.—LC-MS ultra, eluent additive for UHPLC-MS. CAS No. 540-69-2 (e.g., Fluka art. 14266; www.sigmaaldrich.com).

(*6*) *Sulfuric acid*.—Concentrated, *w* = 95–97%. CAS No. 7664-93-9 (e.g., Merck art. 100731; www.merckmillipore.com).

(*7*) *Formic acid*.—Concentrated (e.g., Merck art. 100264; www.chemdat.info). CAS No. 64-18-6.

(*8*) *Sodium fluoroacetate*.—CAS No. 62-74-8, *w* = 99%, 10 µg/mL in water (e.g., Dr. Ehrenstorfer art. DRE-L13772000AL; www.lgcstandards.com).

(*9*) *$^{13}C_2$-Sodium fluoroacetate*.—*w* = 99%, isotopic purity >99.5% (e.g., BDG Synthesis art. 130042-10; http://bdg.co.nz).

(**b**) *Materials*.—

(*1*) *Falcon tubes, conical, polypropylene*.—50 mL (e.g., Becton Dickinson Labware art. 352070; www.bdbiosciences.com).

(*2*) *Falcon tubes, conical, polypropylene*.—15 mL (e.g., Becton Dickinson Labware art. 352097; http://www.bdbiosciences.com).

(*3*) *Centrifuge with rotors adapted for 50 and 15 mL tubes*.—4000 × *g*, temperature controlled (e.g., Multifuge Heraeus; www.thermo.com).

(*4*) *Vortex*.—e.g., Millian Genie 2 (http://www.milian.com).

(*5*) *Centrifuge with rotor adapted for 2 mL tubes*.—17 000 × *g* (e.g., Heraeus Frisco 17; www.thermoscientific.com).

(*6*) *Microcentrifuge tubes, polypropylene*.—2 mL (e.g., Trefflab art. 9607246901; www.treff-ag.ch).

(*7*) *Analytical balance*.—With precision range 0.01 mg.

(*8*) *Shaker*.—GenoGrinder Model 2010 (www.spexsampleprep.com).

(*9*) *Evaporator*.—e.g., Reacti-Vap Evaporator (art. TS-18825; www.thermo.com).

(**c**) *Special equipment and instrumentation*.—Where a specific model is cited, an alternative may be used if it has the same characteristics.

(*1*) *HPLC system*.—Agilent 1200 SL (www.agilent.com) coupled to a Sciex 5500 triple stage quadrupole mass spectrometer equipped with a TurboIonSpray® ionization source (www.sciex.com).

(*2*) *HPLC column*.—Acquity UPLC BEH Amide, 2.1 × 100 mm, 1.7 µm (Waters art. 186004801; www.waters.com).

(**d**) *Glassware decontamination*.—No specific requirement.

C. Preparation of Reagents

Volumes of glassware are purely indicative and may be modified as long as the proportion of reagents is maintained.

(**a**) *Sodium fluoroacetate stock standard solution, 10 µg/mL in water*.—The stock standard solution is available as ready-to-use 10 mL solution. Store at room temperature for the time given in the certificate of analysis.

(**b**) *Sodium fluoroacetate working standard solution, 1.0 µg/mL in acetonitrile–water (9 + 1)*.—Into a 10 mL volumetric flask, pipet 1.0 mL of the stock standard solution 10 µg/mL, **C(a)**. Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**c**) *Sodium fluoroacetate working standard solution, 0.2 µg/mL in acetonitrile*.—Into a 10 mL volumetric flask, pipet 2.0 mL of the stock standard solution 1 µg/mL, **C(b)**. Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**d**) *Sodium fluoroacetate working standard solution, 0.05 µg/mL in acetonitrile*.—Into a 10 mL volumetric flask, pipet 2.5 mL of the stock standard solution 0.2 µg/mL, **C(c)**. Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**e**) *$^{13}C_2$-Sodium fluoroacetate (IS) stock standard solution, 1000 µg/mL in water*.—Into a 10 mL volumetric flask, weigh 10 ± 0.1 mg of standard. Dissolve and complete to the mark with water.

Alternatively (to minimize analyst exposure during weighing) weigh the container containing the analyte first (w_1, in mg), then transfer its whole content into a 10 mL volumetric flask. Dissolve and complete to mark with water for chromatography. Weigh again the empty original container once dried (w_2, in mg). Concentration of this solution in µg/mL is $1000 \times (w_1 - w_2)/10$. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**f**) *$^{13}C_2$-Sodium fluoroacetate (IS) working standard solution, 10 µg/mL in acetonitrile*.—Into a 10 mL volumetric flask, pipet 100 µL of the stock solution 1000 µg/mL, **C(e)**. Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**g**) *$^{13}C_2$-Sodium fluoroacetate (IS) working standard solution, 0.2 µg/mL in acetonitrile*.—Into a 50 mL volumetric

Table 2015.03A. Pipetting schema for the calibration curve

	Standard					
	1	2	3	4	5	6
Working standard solution of sodium fluoroacetate, 0.2 µg/mL, **C**(**c**), µL	0	50	150	300	500	1000
Working standard solution of IS, 0.2 µg/mL, **C**(**g**), µL	500	500	500	500	500	500
Acetonitrile	Complete to the 5 mL mark					
This corresponds to:						
Concentration of sodium fluoroacetate, ng/mL	0	2	6	12	20	40
Concentration of IS, ng/mL	20	20	20	20	20	20

flask, pipet 1000 µL of the working standard solution 10 µg/mL, **C**(**f**). Complete to volume with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**h**) *Standard solutions for calibration curve*.—Into six separate 5 mL volumetric flasks, transfer the volumes of working standard solutions as described in Table **2015.03A**. Complete to the mark with acetonitrile. Store at –20°C for no longer than 6 months. Allow warming at room temperature before use.

(**i**) *Solutions for LC-MS/MS*.—

(*1*) *Mobile phase A, water containing 5 mM ammonium formate and 0.01% (v/v) formic acid*.—Into a weighing boat, weigh 315 ± 5 mg ammonium formate. Transfer this mass into a 1000 mL volumetric flask. Add approximately 300 mL water for chromatography and mix to dissolve. Add 100 µL concentrated formic acid. Complete to volume with water for chromatography. Mix. Store at room temperature for no longer than 1 month.

(*2*) *Mobile phase B, acetonitrile*.—Use acetonitrile hyper grade for LC-MS.

(*3*) *Solution for flushing injection port, acetonitrile–water (1 + 1)*.—Into a 1000 mL volumetric flask, transfer by means of graduated cylinder, 500 mL of acetonitrile gradient grade for chromatography. Complete to volume with water for chromatography. Transfer into an HPLC bottle. Store at room temperature for no longer than 1 month.

D. Sampling and Preparation of Test Samples

(**a**) *Sampling procedure*.—A representative sample (minimum 100 g or 100 mL) should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

(**b**) *Laboratory sample*.—Store in the laboratory at room temperature until analysis, unless otherwise mentioned.

(**c**) *Test sample preparation*.—

(*1*) *Powdered sample*.—Mix well the powdered laboratory sample by means of a spoon before taking a test portion. Alternatively, transfer the whole sample into a container of capacity about twice that of the laboratory sample volume. Close the container immediately. Mix thoroughly by repeatedly shaking and inverting the container.

(*2*) *Liquid sample*.—Shake thoroughly the container containing the sample.

E. Preparation of Test Portions and Extraction Procedure

QC samples (certified, *P*-test, in-house reference samples, or spiked samples) must be regularly included and analyzed in duplicate. Different product types should be analyzed regularly in duplicate.

If necessary, different sized glassware may be substituted for specific volumes listed during the preparation of test solutions as long as the proper dilutions ratios are maintained.

(**a**) *Test portion preparation*.—

(*1*) *Powdered sample*.—Into a 50 mL polypropylene Falcon tube, weigh 5.0 ± 0.1 g powdered sample, **D**(**c**). Record the mass to 0.1 g.

Add 20 mL water for chromatography. Mix thoroughly by inversion and place onto a GenoGrinder shaker. Shake for 1.5 min at 1500 rpm. No lump should be visible.

Transfer 5.0 ± 0.1 g of this slurry into a 15 mL polypropylene Falcon tube. Record the mass to 0.1 g.

Add 50 µL of the IS working solution 0.2 µg/mL, **C**(**g**). Mix thoroughly and make sure that the spiked volume is totally absorbed by the matrix. This spike corresponds to 10 µg/kg equivalent-in-sample concentration of IS.

(*2*) *Liquid sample*.—Into a 15 mL polypropylene Falcon tube, weigh 5.0 ± 0.1 g of liquid sample, **D**(**c**).

Add 250 µL of the IS working solution 0.2 µg/mL, **C**(**g**). Mix thoroughly and make sure that the spiked volume is totally absorbed by the matrix. This spike corresponds to 10 µg/kg equivalent-in-sample concentration of IS.

(**b**) *Extraction procedure*.—To the test portion prepared as described in **E**(**a**)(*1*) or **E**(**a**)(*2*), add 8 mL acetonitrile. Mix thoroughly. Place onto a GenoGrinder shaker and shake for 1.5 min at 1500 rpm.

Centrifuge at 4000 × *g* at room temperature for 5 min and transfer the supernatant (approximately 9 to 10 mL) into a 50 mL Falcon tube.

Add 10 mL hexane. Place onto a GenoGrinder shaker and shake for 1.5 min at 1500 rpm.

Centrifuge at 4000 × *g* at room temperature for 5 min. Pipet the upper hexane phase and discard it to waste.

Add 100 µL of concentrated sulfuric acid (H_2SO_4) to the solution containing the analyte. Mix thoroughly. The resulting pH must be ≤1 to have the analyte in its acidic form (pKa of fluoroacetic acid is 2.39).

Add a buffer salt mixture (Agilent QuEChERS ready-to-use mix) containing 4.0 ± 0.4 g $MgSO_4$ and 1.0 ± 0.1 g NaCl. Immediately hand-shake by inversion or by vortexing to prevent any lump formation. Place onto a GenoGrinder shaker and shake for 1.5 min at 1500 rpm.

Centrifuge at 4000 × *g* at room temperature for 5 min and transfer the supernatant (approximately 5 mL) into a 15 mL Falcon tube.

Evaporate the collected supernatant under a stream of nitrogen at 40 ± 2°C until a 0.5 mL remaining volume. A mark at the 0.5 mL level is visible onto the tube. Do not evaporate to lower volumes to prevent loss on evaporation.

Transfer the 0.5 mL remaining volume into a 2 mL tube and centrifuge at 17 000 × *g* at room temperature for 5 min.

Transfer the clear supernatant into an HPLC vial for further LC-MS/MS analysis.

(**c**) *Reagent blank*.—In order to control any contamination during the sample workup, a reagent blank must be analyzed

Table 2015.03B. HPLC conditions for the analysis of sodium fluoroacetate

Mobile phase A	Water containing 5 mM ammonium formate and 0.01% formic acid, **C**(**i**)(*1*)
Mobile phase B	Acetonitrile, **C**(**i**)(*2*)
Injection volume	20 µL
Column	Waters Acquity UPLC BEH Amide, 2.1 × 100 mm, 1.7 µm
Column oven temp.	45°C
Flow rate	0.45 mL/min
Needle wash	In flush port for 20 s using acetonitrile–water (1 + 1) solution, **C**(**i**)(*3*)
Diverter valve	HPLC flow is directed into the MS detector between 1.0 and 2.5 min
Gradient	LC gradient is described in Table **2015.03C**

along with each series of routine samples. Water is used instead of milk. Proceeded exactly as described in **E**(**a**) and (**b**).

F. Instrumental Conditions

(**a**) *LC-MS/MS analysis*.—Where a specific instrument is cited, an alternative may be used provided it has the same or better characteristics. As well, an alternative HPLC column may be used provided it allows a retention time of the eluting analyte that is at least twice the retention time corresponding to the void volume of the column.

(*1*) *HPLC conditions*.—Using an Agilent 1200 SL HPLC system (*see* Table **2015.03B**). *See* Table **2015.03C** for LC gradient.

Using these conditions, the compound elutes at approximately 1.7 min (*see* Figures **2015.03B**–**E**).

(*2*) *MS parameters*.—MS parameters (Tables **2015.03D** and **E**) are obtained by separately syringe-infusing standard solution (approximately 1 µg/mL) of each unlabeled and labeled compounds (syringe flow rate of 10 µL/min) along with the HPLC flow at 0.45 mL/min using a T connector. The HPLC flow is constituted with 10% A, **C**(**i**)(*1*), and 90% B, **C**(**i**)(*2*).

(**b**) *Instrument check test*.—Before routine analysis, ensure that the LC-MS/MS apparatus is working in conditions such as the method remains fit for purposes. This involves to inject a low concentration calibrant [e.g., STD 2, **C**(**h**)] to check that sensitivity of the instrument is adequate.

G. Operating Procedure and Determination

(**a**) *Sequence setup*.—Inject solutions in the following order: acetonitrile (as blank solvent) at least three times, standard solutions, **C**(**h**), acetonitrile at least three times, reagent blank, **E**(**c**), extract solutions, **E**(**b**), and standard solutions, **C**(**h**), again. Inject acetonitrile after each three to four extract solutions to check for any carry-over.

(**b**) *Calibration*.—Draw a calibration curve by plotting peak area ratio of the analyte and its IS (= *y* axis) against concentration ratio of the analyte and its IS (= *x* axis). Calculate the slope and intercept by linear regression. Check the linearity of the calibration [regression coefficient R^2 should be higher than 0.98 and relative standard deviation of the average of response factors (= *y*/*x*) should be <15%].

(**c**) *Identification and confirmation*.—Sodium fluoroacetate is identified and confirmed when the following criteria are fulfilled (1).

(*1*) The ratio of the chromatographic retention time of the analyte to that of its IS, i.e., the relative retention time, corresponds to that of the averaged relative retention time of the calibration solutions within a ±2.5% tolerance.

(*2*) The peak area ratios from the different transition reactions recorded for the analyte and its IS are within the tolerances fixed by the EU criteria (1) as shown in Table **2015.03E**.

(**d**) *Time of analysis*.—Following this procedure, 20 samples can be analyzed within 24 h.

H. Calculations and Expression of Results

(**a**) *Calculation*.—Calculate the mass fraction, *w*, of sodium fluoroacetate in microgram per kilogram of sample (µg/kg), using the equation:

$$w = \frac{\left(\frac{A_a}{A_{is}}\right) - I}{S} \times \frac{m_{is}}{m_a}$$

where A_a = peak area of the analyte in the sample (transition reaction used for quantification); A_{is} = peak area of the IS in the sample (transition reaction used for quantification); I = intercept of the regression line for the transition reaction used for quantification; S = slope of the regression line for the transition reaction used for quantification; m_{is} = mass of IS added to the test portion, in ng (i.e., 10 ng for powdered sample and 50 ng for liquid sample); m_a = mass of the test portion, in g (i.e., 1 g for powdered sample and 5 g for liquid sample).

(**b**) *Expression of results*.—Report the result of sodium fluoroacetate in µg/kg with one significant figure. Nondetected amount must be expressed as <1 µg/kg.

I. Performance Characteristics

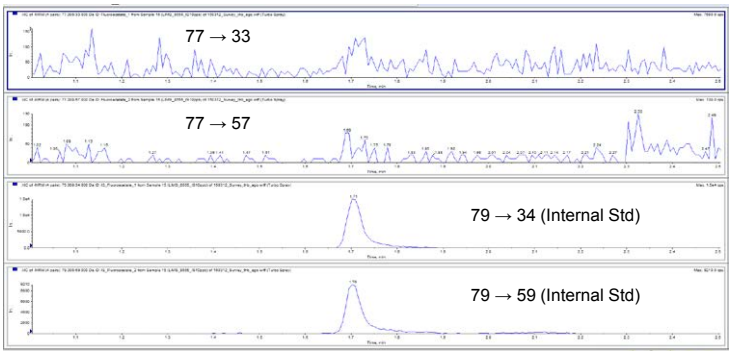
The method was validated using samples provided by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). Infant formulae considered are described in Table **2015.03F**.

Validation was performed according to the protocol described in SANCO/12571/2013 (2): precision data were obtained by spiking each sample at 1 and 10 µg/kg concentration levels (respectively corresponding to 1 × LOQ and 10 × LOQ level). At least two operators were involved in these experiments, each performing five replicates at the mentioned fortification levels on two different days (leading to a total of 10 separate experiments for each fortification level). Nonfortified samples were analyzed as well to verify absence of the pesticide before fortification trials.

Table 2015.03C. LC gradient used for analysis of sodium fluoroacetate

Time, min	A, %	B, %
0	10	90
2.0	10	90
3.0	60	40
4.5	60	40
4.6	10	90
8.0	10	90

Milk Infant Formula
Lactose Free
LIMS 890000008555
Unspiked



Milk Infant Formula
Lactose Free
LIMS 890000008555
Spiked at 1 µg/kg

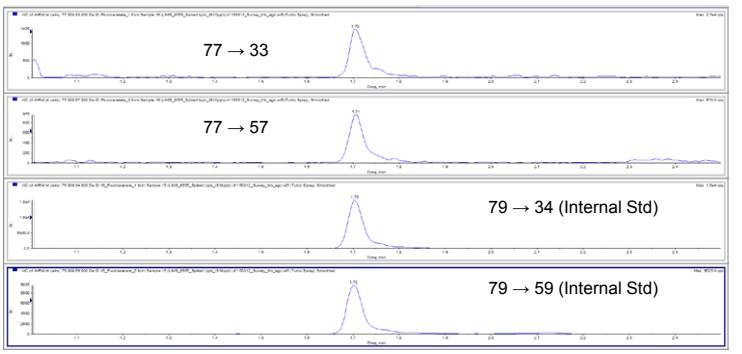
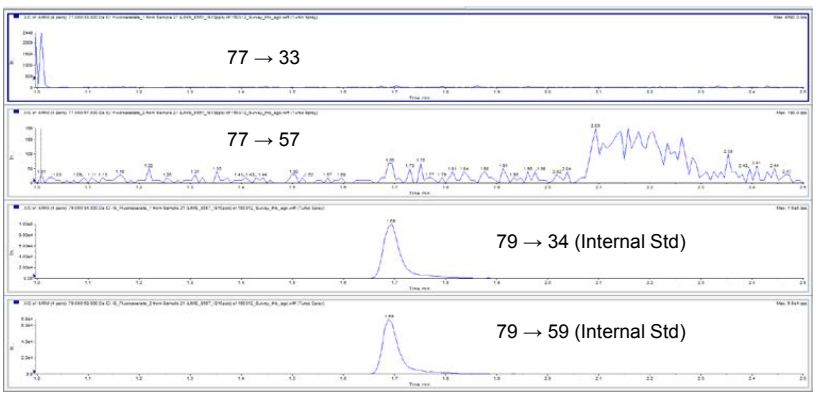


Figure 2015.03B. LC-MS/MS chromatograms of a powdered milk-based infant formula (lactose free) unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Milk Infant Formula
Ready-to-feed (liquid)
LIMS 890000008557
Unspiked



Milk Infant Formula
Ready-to-feed (liquid)
LIMS 890000008557
Spiked at 1 µg/kg

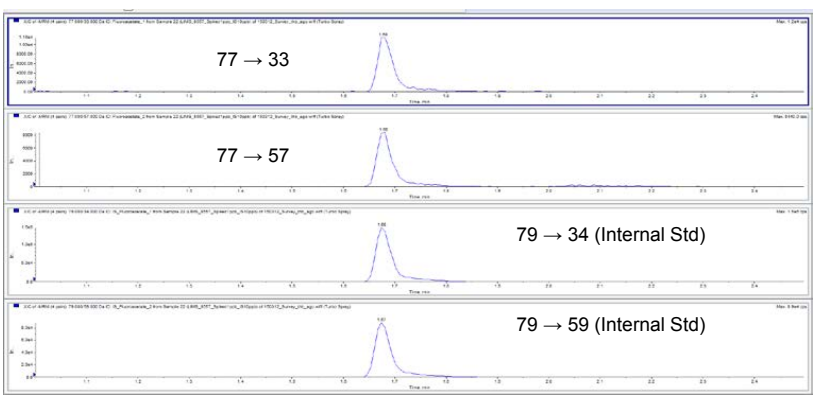
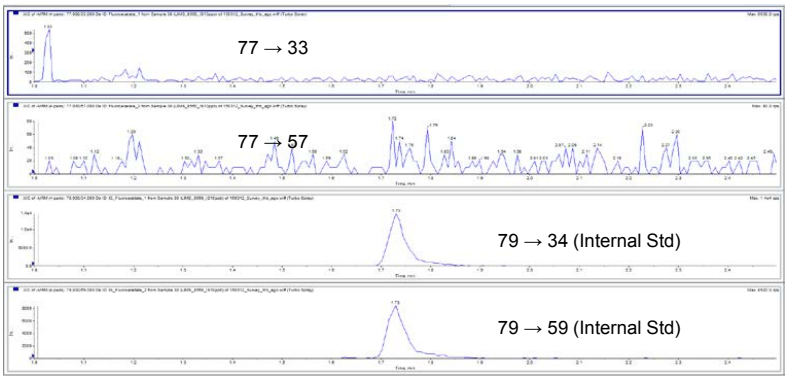


Figure 2015.03C. LC-MS/MS chromatograms of a liquid milk-based infant formula (ready-to-feed) unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Infant Formula
Soya-based
LIMS 890000008560
Unspiked



Infant Formula
Soya-based
LIMS 890000008560
Spiked at 1 µg/kg

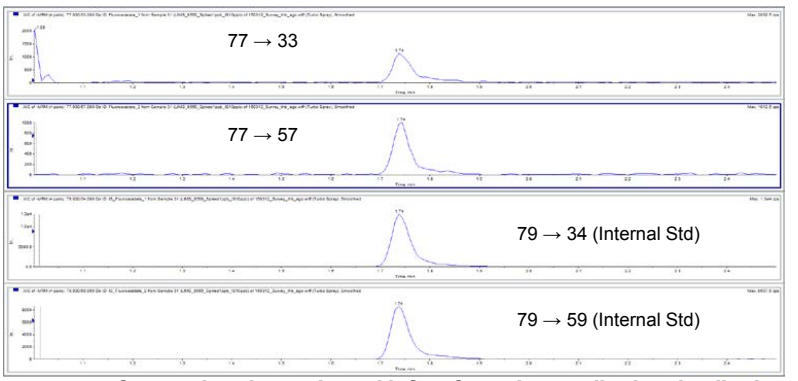
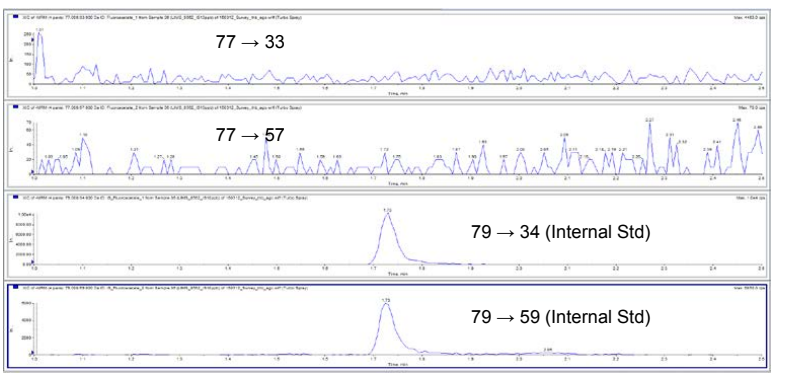


Figure 2015.03D. LC-MS/MS chromatograms of a powdered soya-based infant formula unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Milk Infant Formula
LIMS 890000008562
Unspiked



Milk Infant Formula
LIMS 890000008562
Spiked at 1 µg/kg

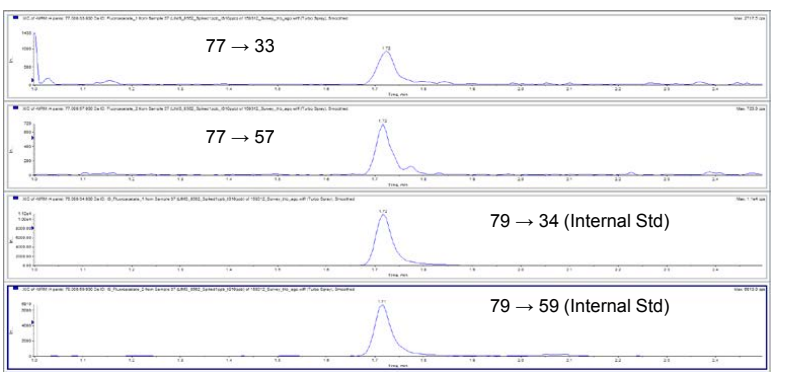


Figure 2015.03E. LC-MS/MS chromatograms of a powdered milk infant formula unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Table 2015.03D. Typical MS parameters for the analysis of sodium fluoroacetate

Parameter	Applied Biosystems Sciex 5500
Ionization type	Electrospray (ESI)
Polarity	Negative ionization
Spray voltage	–4500 V
Source block temperature	500°C
Gas	Curtain gas: 30 psi Ion source gas 1 (GS1): 40 psi Ion source gas 2 (GS2): 40 psi
Source position adjustments	Vertical micrometer value: 5.0 Horizontal micrometer value: 5.0 Electrode protusion: 1.0 mm
Collision energy (CE)	–15
Entrance potential (EP)	–10 V
Collision exit potential (CXP)	–9 V
Declustering potential (DP)	–45 V
CAD gas pressure (MRM)	Medium (6)
Resolution	High on each quadrupole
Scan time (for each transition)	100 ms

(**a**) *Linearity*.—Linearity was verified over the 0–2 area ratio range, corresponding to 0–0.8 ng sodium fluoroacetate (0.4 ng IS) injected on-column. The calibration follows a linear model with $R^2 > 0.99$ and relative standard deviation of the average of response factors <15%.

(**b**) *LOQ*.—LOQ is 1 µg/kg for infant formulae. This concentration corresponds to the lowest fortification level considered during validation.

(**c**) *Recovery, repeatability, and intermediate reproducibility precisions*.—Precision data are described in Table **2015.03G**. All performance data fulfilled SANCO/12571/2013 requirements, i.e., both CV(r) and CV(iR) ≤ 20% and recoveries within the 70–120% range. Exception was recovery for a milk-based formula at the 1 µg/kg fortification level (134%). A small peak was observed at the transition reaction used for quantification in the related unfortified sample extract, meaning that sodium fluoroacetate might be present at a concentration well below the quantification capability of this method. This may explain this higher recovery value.

Table 2015.03F. Samples considered for the validation (SPIFAN kit)

Infant formulae	Batch	Manufacturer (USA)
Milk-based	K16NTAV	PBM Nutritionals
Soy-based	E10NWZC	PBM Nutritionals
Partially hydrolyzed milk-based	410057652Z	Nestlé
Partially hydrolyzed soy-based	410457651Z	Nestlé
High-fat nutritional	00729RF00	Abbott Nutrition
High-protein nutritional	00730RF00	Abbott Nutrition

J. Internal Control Plan

QC samples (certified, *P*-test, in-house reference samples, or spiked samples) must be regularly included and analyzed in duplicate.

Spiked experiment.—Spike the test portion with 20 µL (for powdered matrices) or 100 µL (for liquid matrices) of standard working solution 0.05 µg/mL, **C**(**d**), at the same time as the IS is added, **E**(**a**). This corresponds to 10 µg/kg spiking level. Calculate the recovery rate (Rec) of the spiked sample using the following equation:

$$\mathrm{Rec} = \frac{\rho_T - \rho_N}{\rho_{Spiked}} \times 100$$

where ρ_T is the total concentration of sodium fluoroacetate measured in the spiked sample in micrograms per kg. ρ_N is the native concentration of sodium fluoroacetate measured

Table 2015.03E. Transition reactions monitored for the analysis of sodium fluoroacetate (as its fluoroacetate anion) and its corresponding IS and peak area ratios along with their limit of acceptance according to CD 2002/657/EC (1)

	Transition reactions (*m/z*) used for		
	Quantification	Analyte confirmation	Peak area ratio ± limit, %
Fluoroacetate	77.0 → 33.0[a]	77.0 → 57.0[a]	0.80 ± 20
$^{13}C_2$-Fluoroacetate (IS)	79.0 → 34.0	79.0 → 59.0	0.68 ± 20

[a] *m/z* 57 corresponds to the loss of hydrofluoric acid $[M\text{-}HF]^-$ and *m/z* 33 to the loss of carbone dioxide $[M\text{-}CO_2]^-$.

Table 2015.03G. Performance characteristics

	Recovery, %[a]		CV(r), %		CV(iR), %	
Sample	1 µg/kg	10 µg/kg	1 µg/kg	10 µg/kg	1 µg/kg	10 µg/kg
Milk-based formula	134	105	6	3	11	4
Soy-based formula	117	103	6	2	8	3
Partially Hydrolyzed milk-based formula	111	108	4	5	9	4
Partially hydrolyzed soy-based formula	116	103	9	2	8	2
High-fat nutritional formula	112	101	4	3	7	4
High-protein nutritional formula	96	99	4	2	6	2

[a] Recovery data for sodium fluoroacetate were calculated from values obtained under intermediate reproducibility conditions.

in the nonspiked sample in micrograms per kg. ρ_{Spiked} is the concentration of sodium fluoroacetate spiked in the sample in micrograms per kg (calculated value).

The recovery rate should be between 70–120% when spiked at the 1 µg/kg level.

References

(1) Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, *Off. J. Eur. Commun.* (2002) **L221**, 8–36. http://eur-lex.europa.eu/legal-content/EN/TXT/F/?uri=CELEX:32002D0657&rid=1
(2) SANCO/12571/2013: Guidance document on analytical quality control and validation procedures for pesticide residues analysis in food and feed, http://ec.europa.eu/food/plant/pesticides/guidance_documents/docs/qualcontrol_en.pdf
(3) AOAC SMPR **2015.001** *J. AOAC Int.* (future issue)

AOAC Official Method 2015.04
Monofluoroacetate in Powdered Nutritional Products
Derivatization with 2-Nitrophenylhydrazine and LC-MS/MS
First Action 2015

[Applicable for quantitative determination of monofluoroacetate (MFA) in powdered nutritional product.]

Caution: Monofluoroacetate is highly toxic and volatile below pH 4. Acetonitrile, ethyl acetate, and pyridine are toxic and flammable. Hydrochloric acid is toxic and corrosive.

A. Principle

The method incorporates certain elements from refs (1) and (2). Samples are prepared by dilution in water followed by protein precipitation with acetonitrile. An aliquot of the sample extract is derivatized with 2-nitrophenylhydrazine (2-NPH) in the presence of 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). This reaction achieves the coupling of the carboxyl group of monofluoroacetate to the amino group of 2-NPH with the formation of an amide bond. Samples are processed through a solid-phase extraction (SPE) step in order to exchange sample solvent and achieve a 5-fold concentration of the extract. A stable-isotope labeled internal standard is incorporated into the sample preparation to correct for instrument response and losses in sample preparation. Analysis is performed by ultra-high pressure liquid chromatography (UPLC) coupled to tandem quadrupole mass spectrometry (MS/MS). The MS/MS system is configured to monitor one parent-daughter (precursor-fragment) ion pair for the analyte and internal standard, respectively (*see* Figure **2015.04A**).

B. Apparatus

(**a**) *Balance*.—Analytical.

(**b**) *Balance*.—Micro-analytical.

(**c**) *Beakers*.—Various sizes.

(**d**) *Glass bottles*.—100 and 500 mL.

(**e**) *Graduated cylinders*.—10 and 100 mL.

(**f**) *Flasks, volumetric, glass*.—Class A; 10, 25, and 100 mL.

(**g**) *Vortex mixer*.—Vortex-Genie (Cole-Parmer, http://www.coleparmer.ca), or equivalent.

(**h**) *Centrifuge tubes*.—2, 15, and 50 mL.

(**i**) *Spatulas and scoops*.

(**j**) *Centrifuge*.—Thermo Fisher Scientific (http://www.thermofisher.com) Sorvall Legend XTR, or equivalent.

(**k**) *Glass centrifuge tubes with screw cap*.—VWR (Cat. Nos. 99502-15 and 89001-048, respectively; https://www.vwr.com).

(**l**) *Water bath*.—Thermo Fisher Scientific Precision model 2872, or equivalent.

(**m**) *Glass Pasteur pipets*.

(**n**) *Vacuum manifold for SPE*.

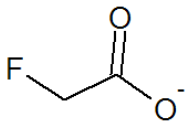


Figure 2015.04A. Monofluoroacetate $C_2H_2FO_2$, MW = 77.03, CAS No. 62-74-8.

Table 2015.04A. Preparation of calibration standards

Standard	Working standard (1 + 10), µL	Working standard, µL	Working internal standard, µL	Acetonitrile–water (60 + 40, v/v), µL
1	10		40	950
2	20		40	940
3	50		40	910
4		10	40	950
5		50	40	910
6		100	40	860

(**o**) *SPE cartridges*.—Supelclean Envi-Chrom P, 500 mg, 6 mL (Cat. No. 57226; Sigma-Aldrich, https://www.sigmaaldrich.com).

(**p**) *SPE cartridge reservoirs*.—25 mL (Supelco; Cat. No. 54258-U).

(**q**) *Positive displacement pipets*.—Gilson (http://www.gilson.com) Microman (M); Model Nos. M10, M100, M250, and M1000. Part Nos. F148501, F148504, F148505, and F148506.

(**r**) *Positive displacement pipet tips*.—Gilson Capillary Piston (CP); Reference Nos. CP10, CP25, CP100, CP250, and CP1000. Part Nos. F148412, F148414, F148014, and F148560.

(**s**) *Plastic syringes*.—B&D 3 mL, or equivalent.

(**t**) *Syringe filters*.—PTFE 0.2 µm, Acrodisc 13 mm, Part No. 28143-930, or equivalent.

(**u**) *Autosampler vials*.—Glass, 2 mL, 12 × 32 mm with screw neck (PTFE pre-split septa caps). Waters (Cat. No. 186000847C; http://www.waters.com), or equivalent.

(**v**) *Vial inserts*.—300 µL (Waters; Cat. No. WAT094170).

(**w**) *Chromatography equipment and supplies*.—(*1*) *Mass spectrometer*.—Waters Xevo TQ-S tandem quadrupole, or equivalent.

(*2*) *Liquid chromatograph*.—Waters Acquity UPLC, or equivalent.

(*3*) *LC column*.—Waters Acquity UPLC® BEH C_{18}, 1.8 µm, 2.1 × 100 mm (Part No. 186002352).

C. Reagents

(**a**) *Sodium fluoroacetate 99.3%*.—Fluka PESTANAL (Cat. No. 31220-100MG; Sigma-Aldrich).

(**b**) *$^{13}C_2$,$^2H_2$-fluoroacetate*.—Cambridge Isotope Laboratories (Cat. No. CDLM-7943-0; http://www.isotope.com).

(**c**) *Acetonitrile*.—Fisher Scientific Optima LC/MS grade (Cat. No. L-1693).

(**d**) *Ethyl acetate*.—Fluka LC/MS Chromasolv (Cat. No. 34972-R).

(**e**) *Ethanol*.—ACS reagent grade (≥99.5%; Sigma-Aldrich, Cat. No. 459844).

(**f**) *Hydrochloric acid*.—Trace metal grade (Fisher Scientific, Cat. No. A508-P500).

(**g**) *Pyridine*.—Sigma-Aldrich (Cat. No. 270970).

(**h**) *Ammonium acetate*.—Fluka (Cat. No. 73594).

(**i**) *2-NPH 97%*.—Sigma-Aldrich (Cat. No. N21588).

(**j**) *EDC*.—Sigma-Aldrich (Cat. No. 03449).

(**k**) *Laboratory water*.—18 MΩ-cm.

D. Solutions

(**a**) *Monofluoroacetate stock standard*.—Approximately 1000 µg/mL in water.

Table 2015.04B. Instrumental conditions

Chromatography					
Flow rate	300 µL/min				
Column temperature	40°C				
Injection volume	10 µL				
Sample temperature	Ambient				
Gradient program	Time	% A	% B	Curve	
	0.00	90	10	6	
	1.00	90	10	6	
	7.00	45	55	6	
	9.00	0	100	6	
	9.01	90	10	6	
	12.00	90	10	6	
MS tune					
Ionization mode	ESI-	Cone gas	250 L/h		
Capillary	0.5 kV	Nebulizer	7.0 bar		
Source offset	20.0 V	Collision gas flow	0.15 mL/min		
Source temperature	150°C	Quad 1 resolution	Unit mass (0.75 Da FWHM)		
Gas temperature	350°C	Quad 2 resolution	Unit mass (0.75 Da FWHM)		
Desolvation gas flow	900 L/h	MS calibration range	50–2000 amu		
MS/MS transitions					
Compound	Parent mass, *m/z*	Daughter mass, *m/z*	Dwell, s	Cone (V)	Collision energy , V
MFA-2NPH	212	182	0.15	20	15
MFA-2NPH_IS	216	186	0.15	20	15

(*1*) Weigh 13.0 ± 1.0 mg sodium fluoroacetate, record weight (*m*) to the nearest 0.1 mg, and transfer to a 10 mL volumetric flask.

(*2*) Dissolve in 4–5 mL laboratory water, dilute to volume with laboratory water, and mix thoroughly.

Store at room temperature. Expiration 6 months.

(*3*) Calculate exact concentration *C* (µg/mL) = *m* × 0.993 × 100 × 0.780 where *m* = amount weighed (mg), 0.993 = purity, and 0.78 = conversion factor from sodium salt to free acid.

(**b**) *Monofluoroacetate working standard*.—8 µg/mL in acetonitrile–water (60 + 40, v/v).

(*1*) Calculate volume of stock standard to be used *V* (µL) = 10 × 1000 × 8/*C*.

(*2*) Transfer *V* µL monofluoroacetate stock standard to a 10 mL volumetric flask.

(*3*) Bring to volume with acetonitrile–water (60 + 40, v/v). Expiration 6 months.

(**c**) *Monofluoroacetate working standard* (*1 + 10*).—0.8 µg/mL in acetonitrile–water (60 + 40, v/v).

(*1*) Transfer 100 µL monofluoroacetate working standard, 8 µg/mL, to 2 mL autosampler vial.

(*2*) Add 900 µL acetonitrile–water (60 + 40, v/v) and mix well. Expiration 6 months.

(**d**) *Internal standard stock solution*.—Approximately 100 µg/mL in water.

(*1*) Transfer the contents of the $^{13}C_2$,$^{2}H_2$-fluoroacetate ampoule (~10 mg) to a 100 mL volumetric flask.

(*2*) Rinse the ampoule three times with laboratory water and transfer each rinse to the volumetric flask.

(*3*) Bring to volume and mix well. Expiration 6 months.

(**e**) *Internal standard working solution*.—Approximately 2.0 µg/mL in acetonitrile–water (60 + 40, v/v).

(*1*) Transfer 20 µL internal standard stock solution to 2 mL autosampler vial.

(*2*) Add 980 µL acetonitrile–water (60 + 40, v/v) and mix well.

(*3*) Scale proportionally as needed for the worklist. Expiration 1 day.

(**f**) *QC1 overspike solution*.—0.5 µg/mL monofluoroacetate in water.

(*1*) Calculate volume of stock standard to be used *V* (µL) = 10 × 1000 × 0.5/*C*.

(*2*) Transfer *V* µL monofluoroacetate stock standard to a 10 mL volumetric flask.

(*3*) Add water to the 10 mL mark and mix well.

(**g**) *QC2 overspike solution*.—10 µg/mL monofluoroacetate in water.

Table 2015.04C. Processing method

Quantitation trace	212 > 182	Smoothing iterations	2
Internal standard trace	216 > 186	Smoothing width	2
Response type	Ratio to IS	Polynomial type	Linear
Predicted RT[a]	4.0 min	Origin	Excluded
RT window	±0.2 min	Weighting	1/X

[a] RT = Retention time.

Table 2015.04D. Typical method performance indicators achieved during in-house validation

Accuracy and precision (n = 9)	10 ng/g	25 ng/g	100 ng/g	500 ng/g
Avg. accuracy, %	116	102	109	100
Precision (RSD), %	3.8	2.4	2.6	3.4

(*1*) Calculate volume of stock standard to be used V (µL) = 10 × 1000 × 10/C.

(*2*) Transfer V µL monofluoroacetate stock standard to a 10 mL volumetric flask.

(*3*) Add water to the 10 mL mark and mix well.

(**h**) *Acetonitrile–water (60 + 40, v/v)*.

(*1*) Transfer 60 mL acetonitrile to 100 mL glass bottle.

(*2*) Add 40 mL water and mix well.

(**i**) *Acetonitrile–water (25 + 75, v/v)*.

(*1*) Transfer 25 mL acetonitrile to 100 mL glass bottle.

(*2*) Add 75 mL water and mix well.

(**j**) *0.1 N HCl–ethanol (50 + 50, v/v)*.

(*1*) Transfer 0.5 mL hydrochloric acid to 100 mL glass bottle.

(*2*) Add 50 mL water.

(*3*) Add 50 mL ethanol and mix well.

(**k**) *Pyridine, 3% in ethanol.*

(*1*) Transfer 3 mL pyridine to 100 mL glass bottle.

(*2*) Add 97 mL ethanol and mix well.

(**l**) *2-NPH*.—Approximately 0.1 M in 0.1 N HCl–ethanol (50 + 50, v/v).

(*1*) Weigh 0.375 ± 0.010 g wet powder into 25 mL volumetric flask.

(*2*) Add ~20 mL 0.1 N HCl–ethanol (50 + 50, v/v).

(*3*) Sonicate and swirl until dissolved.

(*4*) Fill to volume with 0.1 N HCl–ethanol (50 + 50, v/v). Mix well. Expiration 5 days.

(**m**) *EDC*.—0.25 M in 3% pyridine in ethanol.

(*1*) Weigh 1.20 ± 0.05 g EDC in weighing pan.

(*2*) Transfer to suitable storage vessel (minimum 25 mL capacity with a wide mouth).

(*3*) Add 24 mL 3% pyridine in ethanol and mix well. Expiration 5 days.

(**n**) *Mobile phase buffer*.—200 mM ammonium acetate in water.

(*1*) Weigh 1.54 ± 0.05 g ammonium acetate in weighing pan.

(*2*) Transfer to 100 mL volumetric flask with water.

(*3*) Add 50 mL water and swirl until dissolved.

(*4*) Fill to volume with water and mix well. Expiration 5 days.

(**o**) *Mobile phase A*.—10 mM ammonium acetate in water.

(*1*) Transfer 25 mL mobile phase buffer to 500 mL mobile phase bottle.

(*2*) Add 475 mL water and mix well.

(*3*) Sonicate for 5 min. Expiration 5 days.

(**p**) *Mobile phase B*.—10 mM ammonium acetate in acetonitrile–water (95 + 5, v/v).

(*1*) Transfer 25 mL mobile phase buffer to 500 mL mobile phase bottle.

(*2*) Add 475 mL acetonitrile and mix well.

(*3*) Sonicate for 5 min. Expiration 5 days.

(**q**) *Calibration standards (STD 1–6)*.

(*1*) Add volume aliquots (µL) listed in Table **2015.04A** to glass centrifuge tubes. For method blank, use 1 mL acetonitrile–water (60 + 40, v/v).

(*2*) Vortex.

(*3*) Perform steps **E(j)–(m)** concurrently with sample extracts.

(*4*) Transfer 250 µL of each calibration standard solution to 2 mL autosampler vial.

(*5*) Add 750 µL water and mix well. Expiration 48 h. Prepared calibration standards contain 1, 2, 5, 10, 50, and 100 ng/mL, respectively, of derivatized monofluoroacetate along with 10 ng/mL each of derivatized internal standard.

E. Procedure

(**a**) Weigh 1.00 g powdered sample into 50 mL polypropylene centrifuge tube.

(**b**) For QC overspikes add 50 µL of QC1 overspike solution (QC Low, 25 ng/g) or 10 µL of QC2 overspike solution (QC Med, 100 ng/g) or 50 µL of QC2 overspike solution (QC High, 500 ng/g).

(**c**) Add 9 mL water.

(**d**) Shake by hand until homogenous.

(**e**) Transfer 1 mL liquid sample to a 15 mL centrifuge tube.

(**f**) Add 5 µL internal standard working solution and vortex.

(**g**) Add 1.5 mL (2 × 0.75 mL) acetonitrile and shake by hand for 10 s.

(**h**) Centrifuge at 3000 rpm for 5 min at 5°C.

(**i**) Transfer 1 mL of supernatant to glass tube with screw cap.

(**j**) Add 0.5 mL 2-NPH reagent and vortex briefly.

(**k**) Add 0.5 mL EDC reagent and vortex briefly.

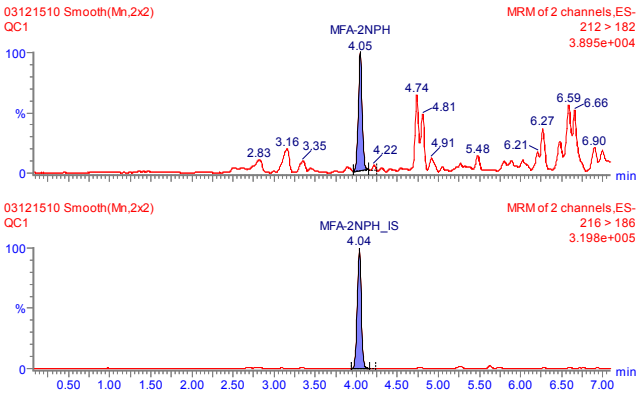


Figure 2015.04B. QC overspike at the method limit of quantitation (10 ng/g powder).

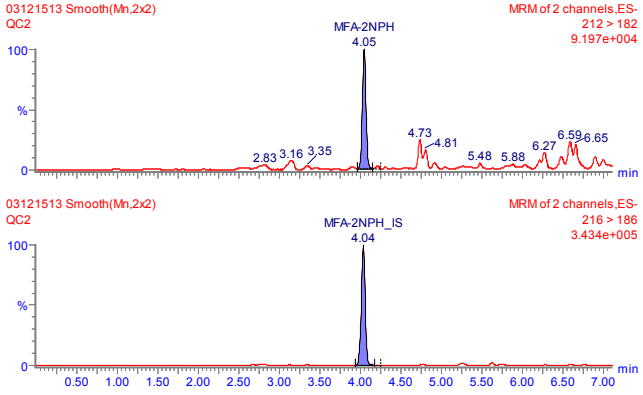


Figure 2015.04C. QC overspike Low at 25 ng/g powder.

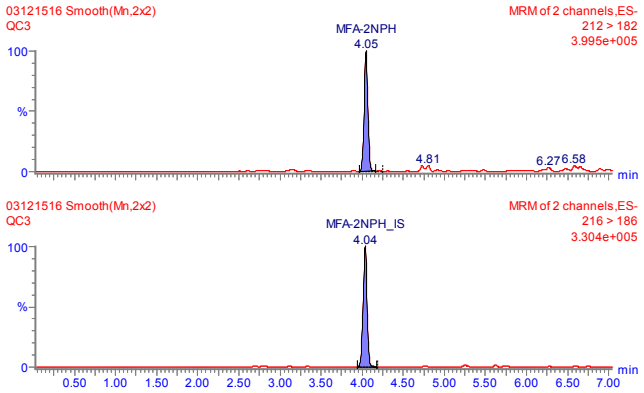


Figure 2015.04D. QC overspike Med at 100 ng/g powder.

(**l**) Cap tightly and incubate in water bath at 80°C for 5 min.

(**m**) Cool to room temperature.

(**n**) Using a glass pipet transfer sample to 50 mL polypropylene centrifuge tube.

(**o**) Add water to a total volume of 15 mL. Cap and invert 10 times.

(**p**) Condition Envi-Chrom P SPE cartridge (500 mg, 6 mL) and reservoir with 10 mL ethyl acetate, 5 mL acetonitrile, and 10 mL water. Leave 1–2 mm water on the cartridge.

(**q**) Load entire sample (15 mL) and allow it to pass through the cartridge.

(**r**) Wash cartridge with 5 mL water.

(**s**) Discard reservoirs.

(**t**) Wash cartridge with 1 mL acetonitrile.

(**u**) Dry cartridge at 5 psi vacuum for 5 min.

(**v**) Elute cartridge with 2 × 5 mL ethyl acetate and collect in 15 mL polypropylene centrifuge tube.

(**w**) Using a glass pipet remove residual aqueous layer from bottom of tube.

(**x**) Evaporate extract to dryness under N_2 gas at 50°C.

(**y**) Reconstitute with 0.4 mL water–acetonitrile (75 + 25, v/v).

(**z**) Vortex for 10 s, sonicate for 1 min, and vortex again for 10 s.

(**aa**) Filter through 0.2 µm PTFE syringe filter into 2 mL centrifuge tube.

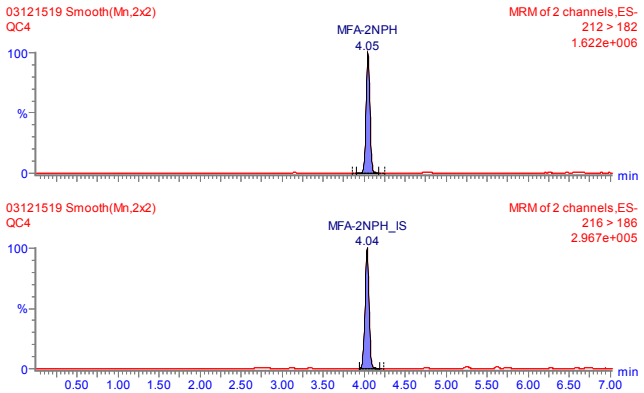


Figure 2015.04E. QC overspike High at 500 ng/g powder.

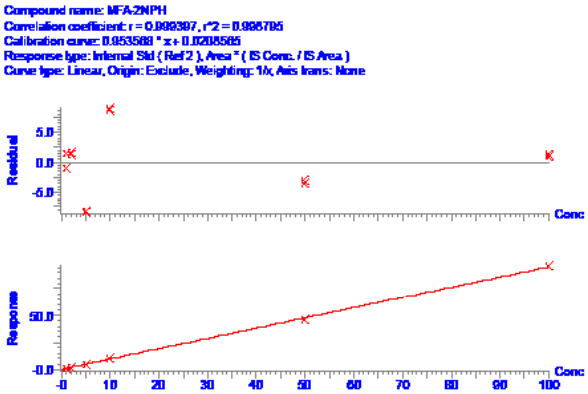


Figure 2015.04F. Calibration curve and residual plot.

(**bb**) Using a 100 µL micropipet transfer 270 µL (3 × 90 µL) extract to autosampler vial with insert.

F. Instrumental Conditions

See Table **2015.04B**.

G. Data Processing

Results are read from the calibration curve and multiplied by 10 (dilution factor from powder to liquid; *see* Table **2015.04C**).

H. Method Acceptance Criteria

(**a**) Calibration curves must have coefficients of determination R^2 of ≥0.99.

(**b**) Calibration curve residuals (relative error) must be ≤15%.

(**c**) Method blank cannot have detectable levels of MFA.

(**d**) QC overspike (apparent) recovery must be within 70–130% of the target value for QC Low, QC Med, and QC High.

I. Demonstrated Method Performance

(**a**) Accuracy of overspiked samples over 3 days and at four different levels ranged between 95–128% during qualification. Table **2015.04D** shows the average recovery and precision at each overspike level.

(**b**) The method detection limit (MDL) and the method quantitation limit (MQL) for MFA in powders are 2 and 10 ng/g, respectively.

J. Example Chromatograms

See Figures **2015.04B–E** for example chromatograms.

K. Example Calibration Curve

See Figure **2015.04F.**

References: (1) *J. Chromatogr. A* **881**, 365(2000)

(2) *J. Chromatogr. A* **1139**, 271(2007)

J. AOAC Int. **98**, 1135(2015)
DOI: 10.5740/jaoac.int.2015.04

AOAC SMPR 2015.001
J. AOAC Int. **98**, 1092(2015)
DOI: 10.5740/jaoac.int.SMPR2015.001

Posted: October 15, 2015

OFFICIAL METHODS

Determination of Monofluoroacetate in Powdered Nutritional Products by Derivatization with 2-Nitrophenylhydrazine and LC-MS/MS: First Action 2015.04

MURALI REDDY

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Submitted for publication March 2015.
Adopted as a First Action Official Method by the Expert Review Panel on Sodium Fluoroacetate and approved by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN).
Approved on: March 16, 2015.
DOI: 10.5740/jaoac.int.2015.04

[Applicable for quantitative determination of monofluoroacetate (MFA) in powdered nutritional product.]

Caution: Monofluoroacetate is highly toxic and volatile below pH 4. Acetonitrile, ethyl acetate, and pyridine are toxic and flammable. Hydrochloric acid is toxic and corrosive.

A. Principle

The method incorporates certain elements from refs (1) and (2). Samples are prepared by dilution in water followed by protein precipitation with acetonitrile. An aliquot of the sample extract is derivatized with 2-nitrophenylhydrazine (2-NPH) in the presence of 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC). This reaction achieves the coupling of the carboxyl group of monofluoroacetate to the amino group of 2-NPH with the formation of an amide bond. Samples are processed through a solid-phase extraction (SPE) step in order to exchange sample solvent and achieve a 5-fold concentration of the extract. A stable-isotope labeled internal standard is incorporated into the sample preparation to correct for instrument response and losses in sample preparation. Analysis is performed by ultra-high pressure liquid chromatography (UPLC) coupled to tandem quadrupole mass spectrometry (MS/MS). The MS/MS system is configured to monitor one parent-daughter (precursor-fragment) ion pair for the analyte and internal standard, respectively (*see* Figure **2015.04A**).

B. Apparatus

(**a**) *Balance*.—Analytical.
(**b**) *Balance*.—Micro-analytical.
(**c**) *Beakers*.—Various sizes.
(**d**) *Glass bottles*.—100 and 500 mL.
(**e**) *Graduated cylinders*.—10 and 100 mL.
(**f**) *Flasks, volumetric, glass*.—Class A; 10, 25, and 100 mL.
(**g**) *Vortex mixer*.—Vortex-Genie (Cole-Parmer, http://www.coleparmer.ca), or equivalent.

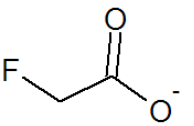

Figure 2015.04A. Monofluoroacetate $C_2H_2FO_2$, MW = 77.03, CAS No. 62-74-8.

(**h**) *Centrifuge tubes*.—2, 15, and 50 mL.

(**i**) *Spatulas and scoops*.

(**j**) *Centrifuge*.—Thermo Fisher Scientific (http://www.thermofisher.com) Sorvall Legend XTR, or equivalent.

(**k**) *Glass centrifuge tubes with screw cap*.—VWR (Cat. Nos. 99502-15 and 89001-048, respectively; https://www.vwr.com).

(**l**) *Water bath*.—Thermo Fisher Scientific Precision model 2872, or equivalent.

(**m**) *Glass Pasteur pipets*.

(**n**) *Vacuum manifold for SPE*.

(**o**) *SPE cartridges*.—Supelclean Envi-Chrom P, 500 mg, 6 mL (Cat. No. 57226; Sigma-Aldrich, https://www.sigmaaldrich.com).

(**p**) *SPE cartridge reservoirs*.—25 mL (Supelco; Cat. No. 54258-U).

(**q**) *Positive displacement pipets*.—Gilson (http://www.gilson.com) Microman (M); Model Nos. M10, M100, M250, and M1000. Part Nos. F148501, F148504, F148505, and F148506.

(**r**) *Positive displacement pipet tips*.—Gilson Capillary Piston (CP); Reference Nos. CP10, CP25, CP100, CP250, and CP1000. Part Nos. F148412, F148414, F148014, and F148560.

(**s**) *Plastic syringes*.—B&D 3 mL, or equivalent.

(**t**) *Syringe filters*.—PTFE 0.2 µm, Acrodisc 13 mm, Part No. 28143-930, or equivalent.

(**u**) *Autosampler vials*.—Glass, 2 mL, 12 × 32 mm with screw neck (PTFE pre-split septa caps). Waters (Cat. No. 186000847C; http://www.waters.com), or equivalent.

(**v**) *Vial inserts*.—300 µL (Waters; Cat. No. WAT094170).

(**w**) *Chromatography equipment and supplies*.—(*1*) *Mass spectrometer*.—Waters Xevo TQ-S tandem quadrupole, or equivalent.

(*2*) *Liquid chromatograph*.—Waters Acquity UPLC, or equivalent.

(*3*) *LC column*.—Waters Acquity UPLC® BEH C_{18}, 1.8 µm, 2.1 × 100 mm (Part No. 186002352).

C. Reagents

(**a**) *Sodium fluoroacetate 99.3%*.—Fluka PESTANAL (Cat. No. 31220-100MG; Sigma-Aldrich).

(**b**) *$^{13}C_2$,$^{2}H_2$-fluoroacetate*.—Cambridge Isotope Laboratories (Cat. No. CDLM-7943-0; http://www.isotope.com).

(**c**) *Acetonitrile*.—Fisher Scientific Optima LC/MS grade (Cat. No. L-1693).

(**d**) *Ethyl acetate*.—Fluka LC/MS Chromasolv (Cat. No. 34972-R).

(**e**) *Ethanol*.—ACS reagent grade (≥99.5%; Sigma-Aldrich, Cat. No. 459844).

(**f**) *Hydrochloric acid*.—Trace metal grade (Fisher Scientific, Cat. No. A508-P500).

(**g**) *Pyridine*.—Sigma-Aldrich (Cat. No. 270970).

(**h**) *Ammonium acetate*.—Fluka (Cat. No. 73594).

(**i**) *2-NPH 97%*.—Sigma-Aldrich (Cat. No. N21588).

(**j**) *EDC*.—Sigma-Aldrich (Cat. No. 03449).

(**k**) *Laboratory water*.—18 MΩ-cm.

D. Solutions

(**a**) *Monofluoroacetate stock standard*.—Approximately 1000 µg/mL in water.

(*1*) Weigh 13.0 ± 1.0 mg sodium fluoroacetate, record weight (*m*) to the nearest 0.1 mg, and transfer to a 10 mL volumetric flask.

(*2*) Dissolve in 4–5 mL laboratory water, dilute to volume with laboratory water, and mix thoroughly.

Store at room temperature. Expiration 6 months.

(*3*) Calculate exact concentration C (µg/mL) = m × 0.993 × 100 × 0.780 where m = amount weighed (mg), 0.993 = purity, and 0.78 = conversion factor from sodium salt to free acid.

(**b**) *Monofluoroacetate working standard*.—8 µg/mL in acetonitrile–water (60 + 40, v/v).

(*1*) Calculate volume of stock standard to be used V (µL) = 10 × 1000 × 8/C.

(*2*) Transfer V µL monofluoroacetate stock standard to a 10 mL volumetric flask.

(*3*) Bring to volume with acetonitrile–water (60 + 40, v/v). Expiration 6 months.

(**c**) *Monofluoroacetate working standard (1 + 10)*.—0.8 µg/mL in acetonitrile–water (60 + 40, v/v).

(*1*) Transfer 100 µL monofluoroacetate working standard, 8 µg/mL, to 2 mL autosampler vial.

(*2*) Add 900 µL acetonitrile–water (60 + 40, v/v) and mix well. Expiration 6 months.

(**d**) *Internal standard stock solution*.—Approximately 100 µg/mL in water.

(*1*) Transfer the contents of the $^{13}C_2$,$^{2}H_2$-fluoroacetate ampoule (~10 mg) to a 100 mL volumetric flask.

(*2*) Rinse the ampoule three times with laboratory water and transfer each rinse to the volumetric flask.

(*3*) Bring to volume and mix well. Expiration 6 months.

(**e**) *Internal standard working solution*.—Approximately 2.0 µg/mL in acetonitrile–water (60 + 40, v/v).

(*1*) Transfer 20 µL internal standard stock solution to 2 mL autosampler vial.

(*2*) Add 980 µL acetonitrile–water (60 + 40, v/v) and mix well.

(*3*) Scale proportionally as needed for the worklist. Expiration 1 day.

(**f**) *QC1 overspike solution*.—0.5 µg/mL monofluoroacetate in water.

(*1*) Calculate volume of stock standard to be used V (µL) = 10 × 1000 × 0.5/C.

(*2*) Transfer V µL monofluoroacetate stock standard to a 10 mL volumetric flask.

(*3*) Add water to the 10 mL mark and mix well.

(**g**) *QC2 overspike solution*.—10 µg/mL monofluoroacetate in water.

(*1*) Calculate volume of stock standard to be used V (µL) = 10 × 1000 × 10/C.

(*2*) Transfer V µL monofluoroacetate stock standard to a 10 mL volumetric flask.

(*3*) Add water to the 10 mL mark and mix well.

(**h**) *Acetonitrile–water (60 + 40, v/v)*.

(*1*) Transfer 60 mL acetonitrile to 100 mL glass bottle.

(*2*) Add 40 mL water and mix well.

(**i**) *Acetonitrile–water (25 + 75, v/v)*.

(*1*) Transfer 25 mL acetonitrile to 100 mL glass bottle.

(*2*) Add 75 mL water and mix well.

(**j**) *0.1 N HCl–ethanol (50 + 50, v/v)*.

(*1*) Transfer 0.5 mL hydrochloric acid to 100 mL glass bottle.

(*2*) Add 50 mL water.

Table 2015.04A. Preparation of calibration standards

Standard	Working standard (1 + 10), µL	Working standard, µL	Working internal standard, µL	Acetonitrile–water (60 + 40, v/v), µL
1	10		40	950
2	20		40	940
3	50		40	910
4		10	40	950
5		50	40	910
6		100	40	860

(*3*) Add 50 mL ethanol and mix well.

(**k**) *Pyridine, 3% in ethanol.*

(*1*) Transfer 3 mL pyridine to 100 mL glass bottle.

(*2*) Add 97 mL ethanol and mix well.

(**l**) *2-NPH.*—Approximately 0.1 M in 0.1 N HCl–ethanol (50 + 50, v/v).

(*1*) Weigh 0.375 ± 0.010 g wet powder into 25 mL volumetric flask.

(*2*) Add ~20 mL 0.1 N HCl–ethanol (50 + 50, v/v).

(*3*) Sonicate and swirl until dissolved.

(*4*) Fill to volume with 0.1 N HCl–ethanol (50 + 50, v/v). Mix well. Expiration 5 days.

(**m**) *EDC.*—0.25 M in 3% pyridine in ethanol.

(*1*) Weigh 1.20 ± 0.05 g EDC in weighing pan.

(*2*) Transfer to suitable storage vessel (minimum 25 mL capacity with a wide mouth).

(*3*) Add 24 mL 3% pyridine in ethanol and mix well. Expiration 5 days.

(**n**) *Mobile phase buffer.*—200 mM ammonium acetate in water.

(*1*) Weigh 1.54 ± 0.05 g ammonium acetate in weighing pan.

(*2*) Transfer to 100 mL volumetric flask with water.

(*3*) Add 50 mL water and swirl until dissolved.

(*4*) Fill to volume with water and mix well. Expiration 5 days.

(**o**) *Mobile phase A.*—10 mM ammonium acetate in water.

(*1*) Transfer 25 mL mobile phase buffer to 500 mL mobile phase bottle.

(*2*) Add 475 mL water and mix well.

(*3*) Sonicate for 5 min. Expiration 5 days.

(**p**) *Mobile phase B.*—10 mM ammonium acetate in acetonitrile–water (95 + 5, v/v).

(*1*) Transfer 25 mL mobile phase buffer to 500 mL mobile phase bottle.

(*2*) Add 475 mL acetonitrile and mix well.

(*3*) Sonicate for 5 min. Expiration 5 days.

(**q**) *Calibration standards (STD 1–6).*

(*1*) Add volume aliquots (µL) listed in Table **2015.04A** to glass centrifuge tubes. For method blank, use 1 mL acetonitrile–water (60 + 40, v/v).

(*2*) Vortex.

Table 2015.04B. Instrumental conditions

Chromatography				
Flow rate	300 µL/min			
Column temperature	40°C			
Injection volume	10 µL			
Sample temperature	Ambient			
Gradient program	Time	% A	% B	Curve
	0.00	90	10	6
	1.00	90	10	6
	7.00	45	55	6
	9.00	0	100	6
	9.01	90	10	6
	12.00	90	10	6

MS tune			
Ionization mode	ESI-	Cone gas	250 L/h
Capillary	0.5 kV	Nebulizer	7.0 bar
Source offset	20.0 V	Collision gas flow	0.15 mL/min
Source temperature	150°C	Quad 1 resolution	Unit mass (0.75 Da FWHM)
Gas temperature	350°C	Quad 2 resolution	Unit mass (0.75 Da FWHM)
Desolvation gas flow	900 L/h	MS calibration range	50–2000 amu

MS/MS transitions					
Compound	Parent mass, *m/z*	Daughter mass, *m/z*	Dwell, s	Cone (V)	Collision energy , V
MFA-2NPH	212	182	0.15	20	15
MFA-2NPH_IS	216	186	0.15	20	15

Table 2015.04C. Processing method

Quantitation trace	212 > 182	Smoothing iterations	2
Internal standard trace	216 > 186	Smoothing width	2
Response type	Ratio to IS	Polynomial type	Linear
Predicted RT[a]	4.0 min	Origin	Excluded
RT window	±0.2 min	Weighting	1/X

[a] RT = Retention time.

(*3*) Perform steps **E(j)**–(**m**) concurrently with sample extracts.

(*4*) Transfer 250 µL of each calibration standard solution to 2 mL autosampler vial.

(*5*) Add 750 µL water and mix well. Expiration 48 h. Prepared calibration standards contain 1, 2, 5, 10, 50, and 100 ng/mL, respectively, of derivatized monofluoroacetate along with 10 ng/mL each of derivatized internal standard.

E. Procedure

(**a**) Weigh 1.00 g powdered sample into 50 mL polypropylene centrifuge tube.

(**b**) For QC overspikes add 50 µL of QC1 overspike solution (QC Low, 25 ng/g) or 10 µL of QC2 overspike solution (QC Med, 100 ng/g) or 50 µL of QC2 overspike solution (QC High, 500 ng/g).

(**c**) Add 9 mL water.

(**d**) Shake by hand until homogenous.

(**e**) Transfer 1 mL liquid sample to a 15 mL centrifuge tube.

(**f**) Add 5 µL internal standard working solution and vortex.

(**g**) Add 1.5 mL (2 × 0.75 mL) acetonitrile and shake by hand for 10 s.

(**h**) Centrifuge at 3000 rpm for 5 min at 5°C.

(**i**) Transfer 1 mL of supernatant to glass tube with screw cap.

(**j**) Add 0.5 mL 2-NPH reagent and vortex briefly.

(**k**) Add 0.5 mL EDC reagent and vortex briefly.

(**l**) Cap tightly and incubate in water bath at 80°C for 5 min.

(**m**) Cool to room temperature.

(**n**) Using a glass pipet transfer sample to 50 mL polypropylene centrifuge tube.

(**o**) Add water to a total volume of 15 mL. Cap and invert 10 times.

(**p**) Condition Envi-Chrom P SPE cartridge (500 mg, 6 mL) and reservoir with 10 mL ethyl acetate, 5 mL acetonitrile, and 10 mL water. Leave 1–2 mm water on the cartridge.

(**q**) Load entire sample (15 mL) and allow it to pass through the cartridge.

(**r**) Wash cartridge with 5 mL water.

(**s**) Discard reservoirs.

(**t**) Wash cartridge with 1 mL acetonitrile.

(**u**) Dry cartridge at 5 psi vacuum for 5 min.

Table 2015.04D. Typical method performance indicators achieved during in-house validation

Accuracy and precision (*n* = 9)	10 ng/g	25 ng/g	100 ng/g	500 ng/g
Avg. accuracy, %	116	102	109	100
Precision (RSD), %	3.8	2.4	2.6	3.4

(**v**) Elute cartridge with 2 × 5 mL ethyl acetate and collect in 15 mL polypropylene centrifuge tube.

(**w**) Using a glass pipet remove residual aqueous layer from bottom of tube.

(**x**) Evaporate extract to dryness under N_2 gas at 50°C.

(**y**) Reconstitute with 0.4 mL water–acetonitrile (75 + 25, v/v).

(**z**) Vortex for 10 s, sonicate for 1 min, and vortex again for 10 s.

(**aa**) Filter through 0.2 µm PTFE syringe filter into 2 mL centrifuge tube.

(**bb**) Using a 100 µL micropipet transfer 270 µL (3 × 90 µL) extract to autosampler vial with insert.

F. Instrumental Conditions

See Table **2015.04B**.

G. Data Processing

Results are read from the calibration curve and multiplied by 10 (dilution factor from powder to liquid; *see* Table **2015.04C**).

H. Method Acceptance Criteria

(**a**) Calibration curves must have coefficients of determination R^2 of ≥0.99.

(**b**) Calibration curve residuals (relative error) must be ≤15%.

(**c**) Method blank cannot have detectable levels of MFA.

(**d**) QC overspike (apparent) recovery must be within 70–130% of the target value for QC Low, QC Med, and QC High.

I. Demonstrated Method Performance

(**a**) Accuracy of overspiked samples over 3 days and at four different levels ranged between 95–128% during qualification. Table **2015.04D** shows the average recovery and precision at each overspike level.

(**b**) The method detection limit (MDL) and the method quantitation limit (MQL) for MFA in powders are 2 and 10 ng/g, respectively.

J. Example Chromatograms

See Figures **2015.04B–E** for example chromatograms.

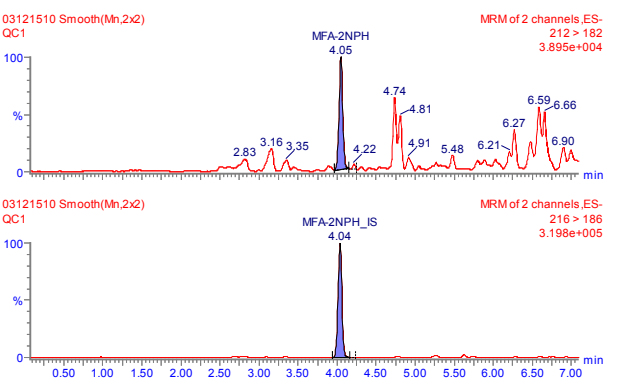

Figure 2015.04B. QC overspike at the method limit of quantitation (10 ng/g powder).

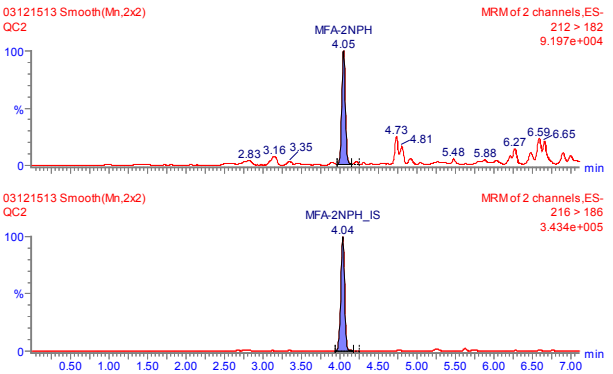

Figure 2015.04C. QC overspike Low at 25 ng/g powder.

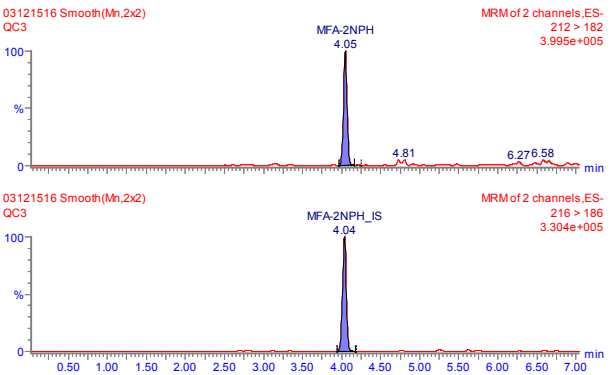

Figure 2015.04D. QC overspike Med at 100 ng/g powder.

K. Example Calibration Curve

See Figure **2015.04F**.

References

(1) *J. Chromatogr. A* **881**, 365(2000)
(2) *J. Chromatogr. A* **1139**, 271(2007)
(3) AOAC SMPR **2015.001** *J. AOAC Int.* (future issue)

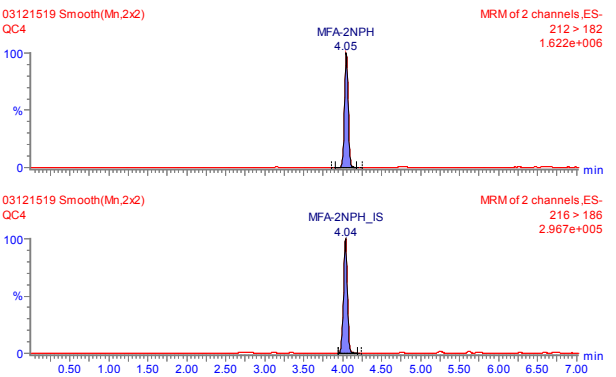

Figure 2015.04E. QC overspike High at 500 ng/g powder.

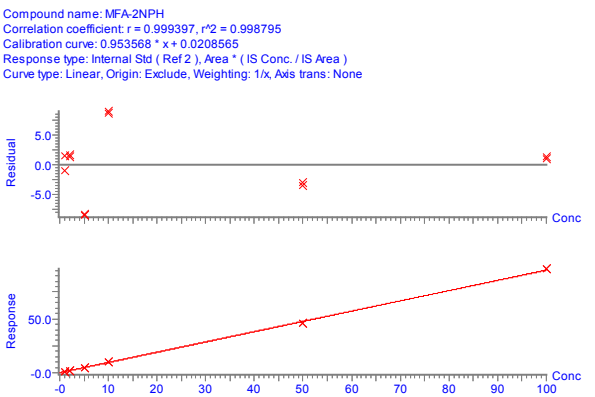

Figure 2015.04F. Calibration curve and residual plot.

AOAC Official Method 2015.05
Partially Hydrolyzed Gluten in Fermented Cereal-Based Products
R5 Competitive ELISA
First Action 2015

[RIDASCREEN® Gliadin competitive ELISA kit is used for the analysis of fermented and hydrolyzed food (e.g., beer, starch syrup, starch, malt extract, sourdough, and soy sauce) that are declared as "gluten-free." The kit is not applicable for measurement of intact gluten.]

Caution: Stop solution contains 0.5 M sulfuric acid; avoid skin and eye contact (*see* Material Safety Data Sheet).

See Table **2015.05** for performance statistics (without outliers) supporting acceptance of the method.

A. Principle

The method is based on an enzyme immunoassay format using a monoclonal antibody that can determine hydrolyzed gluten derived from wheat, rye and barley. The antibody binds to the short amino acid sequence QQPFP and to related sequences, which exist as motifs on all the prolamin subunits (1). Some of these sequences are potentially celiac immuno-stimulatory (2, 3). Since the assay is calibrated to a prolamin hydrolysate mixture form wheat, rye, and barley, a conversion to "gluten" content is achieved by the conversion factor of 2 set by the Codex Alimentarius. No cross-reactivity has been observed to oats, maize, rice, millet, teff, buckwheat, quinoa, or amaranth. Protein fragments for gluten measurement from food are extracted by using ethanol. After centrifugation, the supernatant is used in a competitive method.

The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with a constant amount of gliadin. Standards (mixture of hydrolysates from wheat, rye, and barley prolamins) or sample solutions are pipetted, and peroxidase labeled antigliadin antibody (conjugate with monoclonal R5 antibodies) is added and incubated for 30 min. During incubation, free and immobilized analyte competes for the antibody binding sites (competitive enzyme immunoassay). Any unbound enzyme conjugate is then removed by a washing step. Substrate/chromogen is added to the wells and incubated for 10 min. Bound enzyme conjugate converts the chromogen into a blue product. Addition of the stop solution causes a color change from blue to yellow. The measurement is performed photometrically at 450 nm. The absorption is inversely proportional to the gluten concentration. The response of sample extracts is compared with response observed with calibrators.

B. Apparatus

Apparatus specified here has been tested in the laboratory; equivalent apparatus may be used.

(**a**) *Laboratory mincer/grinder, mortar and pestle, or Ultra-Turrax.*—e.g., Mr. Magic (ds-produkte GmbH, Gallin, Germany).

(**b**) *Rotator or shaker.*—e.g., Roto Shaker Genie (Scientific Industries Inc., Bohemia, NY, USA).

(**c**) *Centrifuge*.—e.g., Minifuge RF (Kendro, Hanau, Germany).

(**d**) *Microtiter plate reader*.—e.g., Tecan Sunrise Remote (Tecan Group, Maennedorf, Switzerland).

(**e**) *Micropipets*.—Variable 20–200 µL and 200–1000 µL.

(**f**) *Graduated pipets*.

(**g**) *Graduated cylinders*.—Up to 1000 mL, plastic or glass.

(**h**) *Centrifugal glass vials with screw tops*.

C. Reagents

Items (**a**)–(**g**) are available as a test kit (RIDASCREEN® Gliadin competitive, R-Biopharm AG, Darmstadt, Germany). All reagents are stable at least over a period of 15 months at 2–8°C (36–46°F) from the date of manufacture. Please refer to the kit label for current expiration.

(**a**) *Microtiter plate*.—Coated with gliadin (96 wells).

(**b**) *Five standard solutions*.—Labeled 0, 20, 60, 180, and 540 ng/mL gluten, 1.3 mL each; ready to use, transparent-capped bottles.

(**c**) *Conjugate.*—Horseradish peroxidase labeled R5 antibody; 0.7 mL, as an 11-fold concentrate, red-capped bottle.

(**d**) *Red Chromogen Pro.*—Substrate/chromogen; 10 mL, ready to use, brown-capped bottle.

(**e**) *Stop solution*.—14 mL, ready to use, yellow-capped bottle.

(**f**) *Sample diluent.*—60 mL, as a 5-fold concentrate, white-capped bottle.

(**g**) *Washing buffer*.—100 mL, as a 10-fold concentrate, brown-capped bottle.

Necessary or recommended but not provided with the test kit:

(**h**) *Distilled water*.

(**i**) *Ethanol*.—99% reagent grade.

(**j**) *Fish gelatin.*—Sigma (St. Louis, MO, USA; Part No. G-7765) or Serva (Heidelberg, Germany; Part No. 22156).

Table 2015.05. Performance statistics for overall competitive R5 ELISA results without outlier (gluten concentrations are shown)

		Sample ID[a]						
	Symbol	1	2	3	4	5	6	7
Total No. of labs	p	13	12	11	13	13	13	13
Total No. of replicates	Sum(n(L))	26	24	22	26	26	26	26
Overall mean of all data (grand mean), mg/kg	XBARBAR	2.36	26.2	119.5	1.29	10.6	48.4	145.6
Repeatability SD, mg/kg	s_r	2.31	7.92	37.2	2.03	1.73	11.2	28.4
Reproducibility SD, mg/kg	s_R	2.98	9.67	37.2	3.05	3.65	12.5	40.0
Repeatability RSD, %	RSD_r	98.0	30.2	31.2	157.3	16.3	23.1	19.5
Reproducibility RSD, %	RSD_R	126.1	36.8	31.2	236.1	34.4	25.9	27.5
Recovery, %		—[b]	87	119	—	—	69	97

[a] *See* Table 1 [*J. AOAC Int.* **98**, 1346(2015)].

[b] — = Not applicable.

D. Standard Reference Material

Not existing today.

E. Standard and Spike Solution

The starting material used for preparation of standard and spike solutions is identical. Wheat, rye, and barley were separately digested by pepsin and trypsin, the peptide fragments were mixed (for preparation of the standard solutions), and the protein content was determined according to Dumas (4). This material was stored at –20°C in lyophilized form until reconstitution. In the case of spiking beer, the hordein digest was used. The material is reconstituted in 60% aqueous ethanol and results in a prolamin concentration of 1 mg/mL. The spike solution is diluted appropriately to the desired concentration. The solution is stable for a maximum of 4 weeks at 2–8°C. The standards as part of the test kit are stabilized in an aqueous solution and are designed to be stable for a minimum of 18 months at 2–8°C. Due to the nature of the standard material, all results are only traceable to this relative anchor point. Determination of trueness is not possible since the material is not a certified reference material. Therefore, the accuracy of the assay system could be biased but is still precise.

F. General Preparation

(**a**) *Sample diluent*.—The sample diluent is provided as a 5-fold concentrate. Only the amount that is actually needed should be diluted with distilled water (e.g., 3 mL concentrate + 12 mL distilled water, sufficient for the dilution of 10 samples). This dilution is stable for 1 day. Make sure that the buffer is not contaminated with gliadin.

(**b**) *60% aqueous ethanol*.—Add 150 mL ethanol to 100 mL distilled water and shake well.

(**c**) *60% aqueous ethanol containing liquid fish gelatin at an amount of 10 g/L (e.g., Serva Part. No. 22156 or Sigma Part. No. G-7765; solid content 45%)*.—Add 30 mL distilled water into a 100 mL graduated cylinder; add 10 g fish gelatin and mix well; add 60 mL ethanol, mix, and adjust pH to 8.5 if necessary. Fill up to 100 mL with distilled water.

(**d**) *Conjugate (peroxidase labeled antibody)*.—The antibody enzyme conjugate is provided as an 11-fold concentrate. Since the diluted enzyme conjugate solution has a limited stability, only the amount that is needed for the subsequent analysis on this day should be reconstituted. Before pipetting, the conjugate concentrate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1 + 10) with distilled water (e.g., 100 µL conjugate concentrate + 1 mL water, sufficient for two microtiter strips). Take care that the water is not contaminated with gliadin.

(**e**) *Washing buffer*.—The washing buffer is provided as a 10-fold concentrate. Before use the buffer has to be diluted 1:10 (1 + 9) with water (i.e., add 100 mL buffer concentrate to 900 mL distilled water). The diluted buffer is stable at 2–8°C (35–46°F) for 4 weeks. Before dilution, dissolve any crystals that may have formed in a water bath at 37°C (99°F).

G. Sample Preparation

(**a**) *General recommendation*.—(*1*) Store samples in a cold, dry room protected from light.

(*2*) Carry out the sample preparation in a room isolated from the ELISA procedure; if only one room is available, consider the high sensitivity of the assay and check for contamination [*see* (*4*) and (*5*) below.]

(*3*) Airborne cereal dust and used laboratory equipment may lead to gliadin contamination of the assay. Therefore, wear gloves during the assay and before starting with the assay.

(*4*) Clean surfaces, glass vials, mincers, and other equipment with 60% ethanol, **F**(**b**), also after use for the next sample.

(*5*) If necessary, check for gliadin contamination of reagents and equipment with the test strips RIDA®QUICK Gliadin (Part. No. R7003).

(*6*) Keep in mind that the solid sample can be inhomogeneous; therefore, grind a representative part of the samples very well and homogenize before weighting.

(*7*) All supernatants obtained after centrifugation can be stored in tightly closed vials in the dark at room temperature (20–25°C/68–77°F) up to 4 weeks.

(**b**) *Homogenize a representative amount of the sample (5–50 g)*.—(*1*) *Solid samples (e.g., starch)*.—Weigh 1 g representative, homogeneous sample and add 10 mL 60% ethanol solution, **F**(**b**).

(*2*) *Liquid food (e.g., starch syrup)*.—Mix 1 mL sample with 9 mL 60% ethanol solution, **F**(**b**).

(*3*) *Beer*.—Mix 1 mL sample with 9 mL 60% ethanol solution containing fish gelatin **F**(**c**). Stir the suspension before and during use.

(*4*) *Malt and hops*.—Mix 1 g sample with 10 mL 60% ethanol solution containing fish gelatin, **F**(**c**). Stir the suspension before and during use.

(**c**) *Further procedure for all samples*.—Mix thoroughly for at least 30 s (vortex) and shake well upside down or rotate on a rotator for 10 min. Centrifuge the sample (2500 × *g* at least) at room temperature (20–25°C/68–77°F) for 10 min. Dilute the supernatant 1:50 (1 + 49) with diluted sample diluent, **F**(**a**), e.g., 20 µL supernatant + 980 µL diluted sample diluent. Use 50 µL/well in the assay (*see* **H**).

H. Determination

(**a**) *General recommendations for good test performance*.—(*1*) This test should only be carried out by trained laboratory employees. The instructions for use must be strictly followed. No quality guarantee is accepted after expiry of the kit (*see* expiry label). Do not interchange individual reagents between kits of different lot numbers.

(*2*) Bring all reagents to room temperature (20–25°C; 68–77°F) before use. The Red Chromogen Pro (substrate/chromogen) is light-sensitive; therefore, avoid exposure to direct light.

(*3*) Return all reagents to 2–8°C (35–46°F) immediately after use. Unused microwells should be returned to their original foil bag. Reseal the bag with the desiccant provided in the bag.

(*4*) Do not allow microwells to dry between working steps.

(*5*) Reproducibility in any ELISA is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the ELISA test procedure.

(*6*) Avoid direct sunlight during all incubations; covering the microtiter plates is recommended.

(*7*) Red Chromogen Pro reaction should be carried out in the dark.

(*8*) Each standard and sample should be analyzed in duplicate.

(*9*) Use also gluten-free and gluten-containing (spiked) samples as test controls.

(**b**) *ELISA testing*.—(*1*) Insert a sufficient number of wells into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions.

(*2*) Add 50 µL of each standard solution or prepared sample, **G**(**b**), to separate wells in duplicate.

(*3*) Add 50 µL of diluted enzyme conjugate, **F**(**d**), mix gently by shaking the plate manually, and incubate for 30 min at room temperature (20–25°C/68–77°F).

(*4*) Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with 250 µL washing buffer **F**(**e**), and pour out the liquid again. Repeat two more times.

(*5*) Add 100 µL Red Chromogen Pro (substrate/chromogen solution; brown cap) to each well. Mix gently by shaking the plate manually and incubate for 10 min at room temperature (20–25°C/68–77°F) in the dark.

(*6*) Add 100 µL stop solution to each well. Mix gently by shaking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 10 min after addition of stop solution.

I. Calculation Interpretation and Test Result Report)

(**a**) *Result calculation*.—Special software RIDA®SOFT Win (Part. No. Z9999) is available and strongly recommended for evaluation of the RIDASCREEN® product line. The calculation should be done using a cubic spline function. Extrapolation is not recommended. The prolamin concentration in an extracted sample is read from the calibration curve and given as ng/mL. To calculate the concentration of prolamins or gluten in a sample, the following equations should be used.

(*1*) *Solid samples*.—

$$\text{Gluten, mg/kg} = \text{gluten concentration in extract, ng/mL} \times 500/1000$$

(*2*) *Liquid samples*.—

$$\text{Gluten, mg/L} = \text{gluten concentration in extract, ng/mL} \times 500/1000.$$

Alternatively, a second order polynomial curve fitting could be used.

(**b**) *Result reporting*.—Results are reported in mg/kg for solid samples or mg/L for liquid samples.

J. Criteria for Acceptance of the Standard Curve

The shape of the standard curve is shown in the quality assurance certificate enclosed in the test kit. Absorbances may vary between different runs (e.g., due to different temperatures or analysts). However, the shape of the standard curve should be similar to the one given in the quality assurance certificate.

Minimum requirements are as follows:

(*1*) OD at 450 nm for standard 1 higher than 0.8.

(*2*) OD values for standards should continuously decrease with higher concentrations, especially when comparing standard 1 (0 ng/mL) and standard 2 (20 ng/mL).

(*3*) An OD value for standard 1 that is much higher than the OD value stated in the certificate could be an indication of errors during pipetting or incubation.

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J. AOAC Int. **98**, 1346(2015)
DOI: 10.5740/jaoacint.CS2015.15

Posted: October 13, 2015

FOOD COMPOSITION AND ADDITIVES

Partially Hydrolyzed Gluten in Fermented Cereal-Based Products by R5 Competitive ELISA: Collaborative Study, First Action 2015.05

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In 2008, the AACC International Protein Technical Committee (now Protein and Enzymes Technical Committee) initiated a collaborative study of a method for determining gluten in fermented products, using an R5 competitive ELISA system. The method has been approved as AACCI Approved Method AACCI 38-55.02. The new method has been validated for testing fermented foods and beverages to determine that they conform to the Codex threshold of 20 mg of gluten/kg in total for gluten-free products. It is recommended that the method be accepted by AOAC as Official First Action.

Gluten is a protein fraction found in wheat, rye, barley, oats, and their crossbred varieties and derivatives thereof, to which some persons are intolerant; it is insoluble in water and NaCl solutions with a concentration of 0.5 M (1, 2). Prolamins are gluten fractions that can be extracted with 40–70% ethanol. The prolamins gliadin, secalin, and hordein are found in wheat, rye, and barley, respectively (1). The prolamin content of gluten is generally taken as 50% (1). In foods labeled as "gluten-free," the gluten level must not exceed 20 mg/kg of food (1–3). Foods processed to reduce their gluten content to a level ranging from 20 to 100 mg/kg may not be labeled "gluten-free"; labeling is regulated on a national level (e.g., could be labeled "very low gluten"). From these regulations, it is obvious that effective test methods are needed to determine the gluten concentration in food, beverages, and raw materials.

The Working Group on Prolamin Analysis and Toxicity (PWG) focused on improving the ELISA methodology for gluten analysis because the existing methods were inadequate with respect to sensitivity and reliability (4). Collaboration between the PWG and the research group headed by Enrique Méndez at the University of Madrid led to improved ELISA methods that use both sandwich and competitive assay systems and are based on the monoclonal R5 antibody. This antibody raised against the ω-type of rye prolamins (ω-secalins) is directed toward the epitope glutamine-glutamine-proline-phenylalanine-proline (QQPFP) in gliadins, hordeins, and secalins. The R5 ELISA is commercially available in two versions, as a sandwich ELISA for intact gluten proteins with at least two binding epitopes and as a competitive ELISA for partially hydrolyzed gluten (gluten peptides), which need only one epitope for binding. While the sandwich ELISA has been studied extensively (4, 5) leading to its approval as AACCI Method 38.50.01 (6, 7) and AOAC *Official Method*℠ **2012.01** (First Action), the competitive R5 ELISA method has not been validated so far. The R5 sandwich ELISA is not as suitable as the competitive ELISA format towards partially hydrolyzed gluten due to the fact that the sandwich ELISA needs two binding sites (8). The competitive assay is the method of choice for measuring partially hydrolyzed gluten in foods.

Scope of the Method

The RIDASCREEN® Gliadin competitive enzyme immunoassay quantitates gluten by measurement of peptide fragments of prolamins from wheat (gliadins), rye (secalin), and barley (hordein). To convert this result to gluten, the conversion factor of 2 set by the Codex Alimentarius is used. The antibody binds to the short amino acid sequence QQPFP and to related sequences, which exist as motifs on all the prolamin subunits (9). Some of these sequences are potentially celiac immuno-stimulatory (10, 11). Samples are extracted by a simple sample preparation and can then be analyzed within 40 min. The standard calibration curve covers gluten concentrations in a sample of 10 to 270 mg/kg. For production of a standard material and for spiking, prolamins (gluten measurement) from rye and barley were isolated and checked for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and RP-HPLC. For wheat, the existing PWG gliadin isolate was used. In a second step, secalins, hordeins, and gliadins were digested with pepsin and trypsin and further characterized by RP-HPLC (8). The protein content of these materials was determined according to the Dumas method.

The calibrators for the R5 competitive ELISA use pepsin-trypsin digested prolamin fractions from wheat, rye, and barley in equal proportion by mass. The multiplication factor of 2 (included in the standards) has been used to convert the prolamin into gluten (1).

Received July 14, 2015.
The method was approved by the Expert Review Panel for Food Allergens-Gluten as First Action.
The Expert Review Panel for Food Allergens-Gluten invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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DOI: 10.5740/jaoacint.CS2015.15

Collaborative Study

Study Design

Following the guidelines of AOAC INTERNATIONAL *Official Methods* (12) and AACC International (13), an international collaborative study was set up to validate the R5 competitive ELISA (R-Biopharm RIDASCREEN® Gliadin competitive R7021; Darmstadt, Germany) for gluten quantitation in fermented foods and beverages as an AACCI Approved Method. The study was carried out as a collaboration between the PWG and AACCI. It was coordinated by Peter Koehler (German Research Center for Food Chemistry; chairman of the PWG and member of the Protein and Enzymes Technical Committee of AACCI) in close collaboration with Clyde Don (chair of the Protein and Enzymes Technical Committee of AACCI).

Collaborators

All laboratories participating in the collaborative study were required to be familiar with immunological tests and, if possible, with competitive ELISA tests. They were advised to use a separate test room for the collaborative study due to the low LOD and the possibility of contamination. To check the samples, test requirements, and documentation and to identify critical points, a precollaborative study with four laboratories within Europe was completed before the full collaborative study. Encouraging results were obtained in the prestudy. Only minor changes in the study design were required, and the full collaborative study proceeded as scheduled. Laboratories were given 6 weeks to perform the analyses (August 1 to September 15, 2011). Sixteen laboratories were selected (designated A to P): one each in Argentina, Austria, Belgium, Canada, Finland, Hungary, Ireland, Italy, New Zealand, Sweden, and Switzerland; two in Germany; and three in the United States.

Description of Samples

The following samples were prepared or obtained for the collaborative study:

(**a**) *Beer*.—Gluten-free.
(**b**) *Beer*.—30 mg/kg gluten (15 mg hordeins/kg).
(**c**) *Beer*.—100 mg/kg gluten (50 mg hordeins/kg).
(**d**) *Starch syrup*.—Gluten-free.
(**e**) *Starch syrup*.—Naturally wheat gluten-contaminated.
(**f**) *Sourdough*.—70 mg/kg gluten (35 mg secalins/kg).
(**g**) *Sourdough*.—150 mg/kg gluten (75 mg secalins/kg).

All ingredients, except barley prolamin hydrolysate, contaminated starch syrup, and rye sourdough, were confirmed to be free of gluten contamination before use by means of the R5 competitive ELISA, which was also used in this collaborative study.

Peptic-Tryptic (PT) Hordein Digest

Grains from the barley cv. "Barke" were milled into white flour (ash content 0.50–0.60% in dry matter) using a laboratory mill and a 0.2 mm sieve. Flour (200 g) was dispersed twice in 600 mL light petroleum (boiling range 40–60°C) and stirred for 30 min at room temperature (RT; approximately 20°C). The solvent was removed, and the residue was air-dried overnight on a filter sheet. A 50 g amount of defatted flour was extracted stepwise with 3 × 200 mL buffer (NaCl concentration: 0.4 M, $KNaHPO_4$ concentration: 0.067 M, pH 7.6) followed by 3 × 200 mL 60% (v/v) aqueous ethanol by homogenizing in a centrifuge vessel for 5 min at RT. Each suspension was centrifuged for 30 min at 3550 × *g* and 4°C, and the supernatants were decanted and combined. The combined ethanol extracts were dialyzed against tap water containing acetic acid at a concentration of 0.01 M and freeze-dried providing the hordein fraction (= barley prolamin). The protein compositions of the hordein fractions were analyzed by SDS-PAGE. The hordein pattern was dominated by the γ-hordeins. C-hordeins were less pronounced, and D-hordeins homologous to high-MW glutenin subunits of wheat were absent. The further characterization by RP-HPLC revealed γ-hordeins at a proportion of 61%, C-hordeins at 35%, and only 5% nonidentified peaks. Therefore, it can be concluded that the protein content (84.3 g/100 g) of this isolate is 95% hordein.

Hordein (0.5 g) was suspended in 10 mL distilled water, and the pH was adjusted to 1.8 with 1.0 M HCl (14). Then, 2.5 mg pepsin (Merck, Darmstadt, Germany; No. 7192) was added, and the suspension was stirred for 4 h at 37°C. After adjusting the pH to 7.8 with 1.0 M NaOH, 2.5 mg trypsin (Merck, No. 24579) was added. After further stirring for 4 h at 37°C the pH was adjusted to 4.5 with 1.0 M HCl and the suspension was centrifuged at 4000 × *g* for 20 min at RT. The supernatant was decanted and freeze-dried, providing the peptic-tryptic (PT) hordein digest. The characterization with SDS-PAGE revealed that proteins with an MW of more than 14 kDa were absent. As expected, RP-HPLC chromatograms showed complex peptide patterns. Protein content of the PT hordein digest was 74.0 ± 0.5% (8). The crude protein contents (N × 5.7) of hordein and the PT hordein digest were determined according to Dumas using an FP-328 combustion instrument (Leco, St. Joseph, MI) and EDTA (N = 9.59%) for calibration.

The PT digest does not represent all hydrolysis processes. There are many additional factors, including temperature and time, that can affect the accuracy of the assay. Users should confirm method performance for their specific processes.

Beer

Beer as a typical fermented product that is analyzed by the R5 competitive ELISA was chosen as a sample. Gluten-free beer ("Beer up," malt´n´more trading GmbH, Grieskirchen, Austria) made from sorghum was used as a zero sample and as base material, which was spiked to a defined hordein concentration with the PT hordein digest. The advantage of this was that samples with exactly defined hordein content determined by an independent analytical method (Dumas analysis) were available. Based on the fact that the N-contents of both the PT hordein digest and the hordein had been determined, the amount of added digest corresponded to the amount of hordein used for its preparation. This was crucial for the determination of the recovery. Briefly, a defined amount of PT hordein digest was

added to the gluten-free beer and stirred for 24 h at RT in order to guarantee a homogeneous distribution in the sample.

Sourdough

A sourdough with defined gluten content was prepared by mixing dried, gluten-free quinoa sourdough with an appropriate amount of dried rye sourdough (both from Ernst Böcker GmbH & Co. KG, Minden, Germany) and shaking overhead for 3 h. The rye sourdough was from an approach in which the company tried to digest as much gluten as possible by lactic acid bacteria (fermentation time 72 h). The starting material was pure rye flour. Two sourdough samples with 70 and 150 mg/kg gluten were prepared. The R5 competitive ELISA was used to determine the gluten content of the rye sourdough (2690 mg/kg gluten) as well as the gluten contents of the quinoa/rye sourdough mixtures, which were used as samples in this study. Since one would expect rye gluten concentrations of about 44 g/kg in rye flour (8), more than 90% of gluten was not any longer detectable by the competitive ELISA after fermentation by lactic acid bacteria.

Starch Syrup

One sample of starch syrup was a commercial gluten-free product ("Stayley® 300 Corn Syrup," Tate & Lyle, London, UK), and the other sample was a wheat starch syrup contaminated with gluten from an anonymous industrial supplier. The gluten contamination was detected by means of the R5 competitive ELISA. The analysis provided a gluten concentration of approximately 10 mg/kg.

Homogeneity of Samples

All samples were checked for homogeneity before they were packaged in air-tight bottles and accepted for the collaborative study. This was done by taking 10 representative 1 g aliquots (1 mL for beer) from 10 different parts of the bulk sample and then analyzing by the R5 competitive ELISA. The CV for the gluten-containing samples was 10.1% or less for sourdough and 18.0% or less for beer. The naturally contaminated starch syrup showed higher variation (±22.3%) due to its low gliadin concentration near the LOQ. All samples were accepted for the collaborative study. Gluten-free samples 1 and 4 were considered homogeneous, because all analyses provided values below the LOQ (<10 mg/kg gluten). Both samples showed optical density (OD) values scattering around the zero calibrator provided (CVs of ODs were around ±6%; n = 10).

Presentation of Samples to Laboratories

Following the AOAC collaborative study guidelines, two independent blinded replicates for each sample were provided to the participating laboratories. Each sample was extracted using 60% (v/v) ethanol and analyzed in duplicate in one analytical run. Fourteen samples were analyzed by each laboratory. The high polyphenol content in the beer samples required a different extraction. These samples were specifically labeled and were extracted with 60% (v/v) ethanol containing 10% (w/v) fish gelatin.

Samples and ELISA kits were shipped to participants at a temperature of about 4°C. Each of the samples was labeled according to the sample code for identification (laboratory code plus number). Participants were requested to return a receipt acknowledgment form to indicate receipt and conditions of the shipped samples. They were also directed to follow the storage advice for samples and kits.

Analysis and Data Reporting

The method was written in AACCI style and was provided to each laboratory with instructions to follow the method as written with no deviations. Laboratories were directed to pay particular attention to cases where samples had to be repeated by further dilution and how dilutions were to be carried out. All OD values had to be recorded in a ready-to-use Excel (Microsoft Corp., Redmond, WA) worksheet. Participants were asked to use the RIDA®SOFT calculation software for cubic spline curve fitting; the software was provided with the kit. Final data from the laboratories were sent to the Study Coordinator.

ELISA Kit and Calculation Software

The R5 competitive ELISA kit (R-Biopharm RIDASCREEN® Gliadin competitive R7021) for the quantitation of gluten in fermented food and the software (RIDA®SOFT Win Z9999) for constructing calibration curves (cubic spline fitting) and calculating gluten concentrations from measured ODs were used.

A cubic spline is a curve constructed of piecewise third-order polynomials that pass through a number (m) of control points. The second derivative of each polynomial is commonly set to zero at the endpoints of the pieces. This provides a boundary condition that completes the system of m-2 equations. It produces a "natural" cubic spline and leads to a simple tridiagonal system that can be solved easily to give the coefficients of the polynomials (15). In this way, a function with a continuous curvature over the entire range is obtained. The third derivative is used as a smoothing factor in the calibration curves to determine the extent of interpolation. Lower factors lead to more approximation, and higher ones (>100) lead to more interpolation of the curve function. The RIDASOFT software uses a factor of 10 000. To minimize boundary effects and allow extrapolation, two additional control points are added to the set of control points as the starting and end points, where the starting point is near zero and set to x(0) = 0.001 and y(0) = OD (lowest Standard 1) and the virtual end point is determined by calculating the linear regression of the other control points by assuming that x(n) has the same distance to x(n-1) as x(1) has to x(0). As the cubic spline model did not provide concentration values for samples below the lowest standard, a second-order polynomial curve fitting model was used to determine values for Samples 1 and 4.

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[RIDASCREEN® Gliadin competitive ELISA kit is used

for the analysis of fermented and hydrolyzed food (e.g., beer, starch syrup, starch, malt extract, sourdough, and soy sauce) that are declared as "gluten-free." The kit is not applicable for measurement of intact gluten.]

Caution: Stop solution contains 0.5 M sulfuric acid; avoid skin and eye contact (*see* Material Safety Data Sheet).

A. Principle

The method is based on an enzyme immunoassay format using a monoclonal antibody that can determine hydrolyzed gluten derived from wheat, rye and barley. The antibody binds to the short amino acid sequence QQPFP and to related sequences, which exist as motifs on all the prolamin subunits (9). Some of these sequences are potentially celiac immuno-stimulatory (10, 11). Since the assay is calibrated to a prolamin hydrolysate mixture form wheat, rye, and barley, a conversion to "gluten" content is achieved by the conversion factor of 2 set by the Codex Alimentarius. No cross-reactivity has been observed to oats, maize, rice, millet, teff, buckwheat, quinoa, or amaranth. Protein fragments for gluten measurement from food are extracted by using ethanol. After centrifugation, the supernatant is used in a competitive method.

The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with a constant amount of gliadin. Standards (mixture of hydrolysates from wheat, rye, and barley prolamins) or sample solutions are pipetted, and peroxidase labeled antigliadin antibody (conjugate with monoclonal R5 antibodies) is added and incubated for 30 min. During incubation, free and immobilized analyte competes for the antibody binding sites (competitive enzyme immunoassay). Any unbound enzyme conjugate is then removed by a washing step. Substrate/chromogen is added to the wells and incubated for 10 min. Bound enzyme conjugate converts the chromogen into a blue product. Addition of the stop solution causes a color change from blue to yellow. The measurement is performed photometrically at 450 nm. The absorption is inversely proportional to the gluten concentration. The response of sample extracts is compared with response observed with calibrators.

B. Apparatus

Apparatus specified here has been tested in the laboratory; equivalent apparatus may be used.

(**a**) *Laboratory mincer/grinder, mortar and pestle, or Ultra-Turrax.*—e.g., Mr. Magic, ds-produkte GmbH, Gallin, Germany.

(**b**) *Rotator or shaker.*—e.g., Roto Shaker Genie (Scientific Industries Inc., Bohemia, NY).

(**c**) *Centrifuge*.—e.g., Minifuge RF, Kendro, Hanau, Germany.

(**d**) *Microtiter plate reader*.—e.g., Tecan Sunrise Remote (Tecan Group, Maennedorf, Switzerland).

(**e**) *Micropipets*.—Variable 20–200 µL and 200–1000 µL.

(**f**) *Graduated pipets*.

(**g**) *Graduated cylinders*.—Up to 1000 mL, plastic or glass.

(**h**) *Centrifugal glass vials with screw tops*.

C. Reagents

Items (**a**)–(**g**) are available as a test kit (RIDASCREEN® Gliadin competitive, R-Biopharm AG). All reagents are stable at least over a period of 15 months at 2–8°C (36–46°F) from the date of manufacture. Please refer to the kit label for current expiration.

(**a**) *Microtiter plate*.—Coated with gliadin (96 wells).

(**b**) *Five standard solutions*.—Labeled 0, 20, 60, 180, and 540 ng/mL gluten, 1.3 mL each; ready to use, transparent-capped bottles.

(**c**) *Conjugate.*—Horseradish peroxidase labeled R5 antibody; 0.7 mL, as an 11-fold concentrate, red-capped bottle.

(**d**) *Red Chromogen Pro.*—Substrate/chromogen; 10 mL, ready to use, brown-capped bottle.

(**e**) *Stop solution*.—14 mL, ready to use, yellow-capped bottle.

(**f**) *Sample diluent.*—60 mL, as a 5-fold concentrate, white-capped bottle.

(**g**) *Washing buffer*.—100 mL, as a 10-fold concentrate, brown-capped bottle.

Necessary or recommended but not provided with the test kit:

(**h**) *Distilled water*.

(**i**) *Ethanol*.—99% reagent grade.

(**j**) *Fish gelatin.*—Sigma, St. Louis, MO; Part No. G-7765 or Serva, Heidelberg, Germany; Part No. 22156.

Table 2015.05. Performance statistics for overall competitive R5 ELISA results without outlier (gluten concentrations are shown)

		Sample ID[a]						
	Symbol	1	2	3	4	5	6	7
Total No. of labs	p	13	12	11	13	13	13	13
Total No. of replicates	Sum(n(L))	26	24	22	26	26	26	26
Overall mean of all data (grand mean), mg/kg	XBARBAR	2.36	26.2	119.5	1.29	10.6	48.4	145.6
Repeatability SD, mg/kg	s_r	2.31	7.92	37.2	2.03	1.73	11.2	28.4
Reproducibility SD, mg/kg	s_R	2.98	9.67	37.2	3.05	3.65	12.5	40.0
Repeatability RSD, %	RSD_r	98.0	30.2	31.2	157.3	16.3	23.1	19.5
Reproducibility RSD, %	RSD_R	126.1	36.8	31.2	236.1	34.4	25.9	27.5
Recovery, %		—[b]	87	119	—	—	69	97

[a] *See* Table 1.

[b] — = Not applicable.

D. Standard Reference Material

Not existing today.

E. Standard and Spike Solution

The starting material used for preparation of standard and spike solutions is identical. Wheat, rye, and barley were separately digested by pepsin and trypsin, the peptide fragments were mixed (for preparation of the standard solutions), and the protein content was determined according to Dumas (8). This material was stored at –20°C in lyophilized form until reconstitution. In the case of spiking beer, the hordein digest was used. The material is reconstituted in 60% aqueous ethanol and results in a prolamin concentration of 1 mg/mL. The spike solution is diluted appropriately to the desired concentration. The solution is stable for a maximum of 4 weeks at 2–8°C. The standards as part of the test kit are stabilized in an aqueous solution and are designed to be stable for a minimum of 18 months at 2–8°C. Due to the nature of the standard material, all results are only traceable to this relative anchor point. Determination of trueness is not possible since the material is not a certified reference material. Therefore, the accuracy of the assay system could be biased but is still precise.

F. General Preparation

(**a**) *Sample diluent*.—The sample diluent is provided as a 5-fold concentrate. Only the amount that is actually needed should be diluted with distilled water (e.g., 3 mL concentrate + 12 mL distilled water, sufficient for the dilution of 10 samples). This dilution is stable for 1 day. Make sure that the buffer is not contaminated with gliadin.

(**b**) *60% aqueous ethanol*.—Add 150 mL ethanol to 100 mL distilled water and shake well.

(**c**) *60% aqueous ethanol containing liquid fish gelatin at an amount of 10 g/L (e.g., Serva, Part. No. 22156 or Sigma Part. No. G-7765; solid content 45%)*.—Add 30 mL distilled water into a 100 mL graduated cylinder; add 10 g fish gelatin and mix well; add 60 mL ethanol, mix, and adjust pH to 8.5 if necessary. Fill up to 100 mL with distilled water.

(**d**) *Conjugate (peroxidase labeled antibody)*.—The antibody enzyme conjugate is provided as an 11-fold concentrate. Since the diluted enzyme conjugate solution has a limited stability, only the amount that is needed for the subsequent analysis on this day should be reconstituted. Before pipetting, the conjugate concentrate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1 + 10) with distilled water (e.g., 100 µL conjugate concentrate + 1 mL water, sufficient for two microtiter strips). Take care that the water is not contaminated with gliadin.

(**e**) *Washing buffer*.—The washing buffer is provided as a 10-fold concentrate. Before use the buffer has to be diluted 1:10 (1 + 9) with water (i.e., add 100 mL buffer concentrate to 900 mL distilled water). The diluted buffer is stable at 2–8°C (35–46°F) for 4 weeks. Before dilution, dissolve any crystals that may have formed in a water bath at 37°C (99°F).

G. Sample Preparation

(**a**) *General recommendation.*

(*1*) Store samples in a cold, dry room protected from light.

(*2*) Carry out the sample preparation in a room isolated from the ELISA procedure; if only one room is available, consider the high sensitivity of the assay and check for contamination [*see* (*4*) and (*5*) below.]

(*3*) Airborne cereal dust and used laboratory equipment may lead to gliadin contamination of the assay. Therefore, wear gloves during the assay and before starting with the assay.

(*4*) Clean surfaces, glass vials, mincers, and other equipment with 60% ethanol, **F**(**b**), also after use for the next sample.

(*5*) If necessary, check for gliadin contamination of reagents and equipment with the test strips RIDA®QUICK Gliadin (Part. No. R7003).

(*6*) Keep in mind that the solid sample can be inhomogeneous; therefore, grind a representative part of the samples very well and homogenize before weighting.

(*7*) All supernatants obtained after centrifugation can be stored in tightly closed vials in the dark at room temperature (20–25°C/68–77°F) up to 4 weeks.

(**b**) *Homogenize a representative amount of the sample (5–50 g)*.

(*1*) *Solid samples (e.g., starch)*.—Weigh 1 g representative, homogeneous sample and add 10 mL 60% ethanol solution, **F**(**b**).

(*2*) *Liquid food (e.g., starch syrup)*.—Mix 1 mL sample with 9 mL 60% ethanol solution, **F**(**b**).

(*3*) *Beer*.—Mix 1 mL sample with 9 mL 60% ethanol solution containing fish gelatin **F**(**c**). Stir the suspension before and during use.

(*4*) *Malt and hops*.—Mix 1 g sample with 10 mL 60% ethanol solution containing fish gelatin, **F**(**c**). Stir the suspension before and during use.

(**c**) *Further procedure for all samples*.—Mix thoroughly for at least 30 s (vortex) and shake well upside down or rotate on a rotator for 10 min. Centrifuge the sample (2500 × *g* at least) at room temperature (20–25°C/68–77°F) for 10 min. Dilute the supernatant 1:50 (1 + 49) with diluted sample diluent, **F**(**a**), e.g., 20 µL supernatant + 980 µL diluted sample diluent. Use 50 µL/well in the assay (*see* **H**).

H. Determination

(**a**) *General recommendations for good test performance*.

(*1*) This test should only be carried out by trained laboratory employees. The instructions for use must be strictly followed. No quality guarantee is accepted after expiry of the kit (*see* expiry label). Do not interchange individual reagents between kits of different lot numbers.

(*2*) Bring all reagents to room temperature (20–25°C; 68–77°F) before use. The Red Chromogen Pro (substrate/chromogen) is light-sensitive; therefore, avoid exposure to direct light.

(*3*) Return all reagents to 2–8°C (35–46°F) immediately after use. Unused microwells should be returned to their original foil bag. Reseal the bag with the desiccant provided in the bag.

(*4*) Do not allow microwells to dry between working steps.

(*5*) Reproducibility in any ELISA is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the ELISA test procedure.

(*6*) Avoid direct sunlight during all incubations; covering the microtiter plates is recommended.

(*7*) Red Chromogen Pro reaction should be carried out in the dark.

(*8*) Each standard and sample should be analyzed in duplicate.

(*9*) Use also gluten-free and gluten-containing (spiked) samples as test controls.

(**b**) *ELISA testing*.

(*1*) Insert a sufficient number of wells into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions.

(*2*) Add 50 µL of each standard solution or prepared sample, **G**(**b**), to separate wells in duplicate.

(*3*) Add 50 µL of diluted enzyme conjugate, **F**(**d**), mix gently by shaking the plate manually, and incubate for 30 min at room temperature (20–25°C/68–77°F).

(*4*) Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with 250 µL washing buffer **F**(**e**), and pour out the liquid again. Repeat two more times.

(*5*) Add 100 µL Red Chromogen Pro (substrate/chromogen solution; brown cap) to each well. Mix gently by shaking the plate manually and incubate for 10 min at room temperature (20–25°C/68–77°F) in the dark.

(*6*) Add 100 µL stop solution to each well. Mix gently by shaking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 10 min after addition of stop solution.

I. Calculation Interpretation and Test Result Report)

(**a**) *Result calculation*.—Special software RIDA®SOFT Win (Part. No. Z9999) is available and strongly recommended for evaluation of the RIDASCREEN® product line. The calculation should be done using a cubic spline function. Extrapolation is not recommended. The prolamin concentration in an extracted sample is read from the calibration curve and given as ng/mL. To calculate the concentration of prolamins or gluten in a sample, the following equations should be used.

(*1*) *Solid samples*

$$\text{Gluten, mg/kg} = \text{Gluten concentration in extract, ng/mL} \times 500/1000$$

(*2*) *Liquid samples*

$$\text{Gluten, mg/L} = \text{Gluten concentration in extract, ng/mL} \times 500/1000.$$

Alternatively, a second order polynomial curve fitting could be used.

(**b**) *Result reporting*.—Results are reported in mg/kg for solid samples or mg/L for liquid samples.

Table 1. Gluten concentrations determined by R5 competitive ELISA by all participating laboratories (raw data)

	Gluten concentration, mg/kg[a]													
	1[b]		2		3		4		5		6		7	
	Repeat													
Lab	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	2.13	5.80	23.6	20.5	111.6	93.9	4.47	7.73	7.60	8.62	46.7	47.2	152.9	170.0
B	1.46	2.66	40.8	13.8	151.4	127.4	2.98	2.13	10.6	5.10	38.8	53.0	163.6	122.8
C	5.30	10.6	34.2	82.2	192.2	107.6	6.12	1.90	12.8	12.6	47.2	67.4	181.4	143.4
D	0.74	1.77	23.8	28.6	175.2	97.6	–3.35	–3.41	9.80	11.0	33.0	60.2	106.4	107.6
E	6.45	20.4	72.4	50.4	24.6	204.0	23.5	17.6	20.4	29.4	68.6	72.8	251.0	244.2
F	–5.46	–3.99	14.6	27.0	124.0	160.0	–5.15	–5.55	9.20	6.80	47.0	51.4	128.8	151.6
G	6.06	4.30	32.4	32.0	216.2	208.2	3.29	–2.34	15.0	14.0	46.8	85.4	192.8	203.0
H	7.02	1.56	44.4	26.2	145.6	32.8	5.79	3.17	20.5	16.1	38.8	31.0	94.6	88.9
I	–0.65	–1.33	22.2	13.8	101.2	64.4	-0.89	–0.62	5.44	4.22	35.8	45.0	118.4	75.0
J	–1.50	1.14	21.2	20.0	121.8	128.8	–0.73	–1.63	7.40	8.00	45.6	58.3	132.9	139.2
K	16.3	14.8	50.0	44.8	216.7	308.6	21.1	9.70	33.5	22.4	87.5	80.0	348.0	30.2
L	1.69	–0.33	39.8	49.0	224.8	228.8	–1.83	3.39	13.2	11.6	64.0	67.2	171.6	244.6
M	–0.66	4.13	19.9	19.3	129.4	133.6	–2.27	–0.62	10.0	8.60	36.1	39.6	161.7	120.4
N	0.04	0.76	34.2	18.4	97.0	108.6	1.84	4.41	10.8	9.20	43.4	44.6	117.6	154.4
O	1.57	0.41	19.1	16.5	110.7	136.6	–0.11	1.26	11.7	8.20	51.3	46.3	152.8	164.6
P	5.96	0.84	25.4	24.8	149.4	111.2	1.54	1.33	12.6	10.8	38.2	46.2	194.8	111.2

[a] The calculation of the concentrations of the gluten-containing samples 2, 3, 5, 6, and 7 was done on the basis of a cubic spline function using the RIDA®SOFT Win software; the statistics of the gluten-free samples 1 and 4 were calculated on the basis of a second-order polynomial function; values for blinded samples are given as repeat 1 or repeat 2.

[b] Sample 1, gluten-free beer; sample 2, beer spiked at 30 mg/kg; sample 3, beer spiked at 100 mg/kg; sample 4, gluten-free starch syrup; sample 5, naturally contaminated wheat starch syrup; sample 6, sourdough containing gluten at 70 mg/kg; and sample 7, sourdough containing gluten at 150 mg/kg.

Table 2. Gluten concentrations determined by R5 competitive ELISA after eliminating laboratories E, F, and K

	Gluten concentration, mg/kg[a]													
	1[b]		2		3		4		5		6		7	
	Repeat													
Lab	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	2.13	5.80	23.6	20.5	111.6	93.9	4.47	7.73	7.6	8.62	46.7	47.2	153.0	170.0
B	1.46	2.66	40.8	13.8	151.4	127.4	2.98	2.13	10.6	5.1	38.8	53.0	163.6	122.8
C	5.30	10.6	34.2[c]	82.2[c]	192.2	107.6	6.12	1.90	12.8	12.6	47.2	67.4	181.4	143.4
D	0.74	1.77	23.8	28.6	175.2	97.6	–3.35	–3.41	9.8	11.0	33.0	60.2	106.4	107.6
G	6.06	4.30	32.4	32.0	216.2[d]	208.2[d]	3.29	–2.34	15.0	14.0	46.8	85.4	192.8	203.0
H	7.02	1.56	44.4	26.2	145.6	32.8	5.79	3.17	20.5	16.1	38.8	31.1	94.6	88.9
I	–0.65	–1.33	22.2	13.8	101.2	64.4	–0.89	–0.62	5.4	4.2	35.8	45.0	118.4	75.0
J	–1.50	1.14	21.2	20.0	121.8	128.8	–0.73	–1.63	7.4	8.0	45.6	58.3	132.9	139.2
L	1.69	–0.33	39.8	49.0	224.8[d]	228.8[d]	–1.83	3.39	13.2	11.6	64.0	67.2	171.6	244.6
M	–0.66	4.13	19.9	19.3	129.4	133.6	–2.27	–0.62	10.0	8.6	36.1	39.6	161.7	120.4
N	0.04	0.76	34.2	18.4	97.0	108.6	1.84	4.41	10.8	9.2	43.4	44.6	117.6	154.4
O	1.57	0.41	19.1	16.5	110.7	136.6	–0.11	1.26	11.7	8.2	51.3	46.3	152.8	164.6
P	5.96	0.84	25.4	24.8	149.4	111.2	1.54	1.33	12.6	10.8	38.2	46.2	194.8	111.2

[a] The calculation of the concentrations of the gluten-containing samples 2, 3, 5, 6, and 7 was done on the basis of a cubic spline function using the RIDA®SOFT Win software; the statistics of the gluten-free samples 1 and 4 were calculated on the basis of a second-order polynomial function; values for blinded samples are given as repeat 1 or repeat 2.

[b] For samples 1–7 *see* Table 1.

[c] Means outlier according to the Cochran test.

[d] Means outlier according to the double Grubbs' test.

J. Criteria for Acceptance of the Standard Curve

The shape of the standard curve is shown in the quality assurance certificate enclosed in the test kit. Absorbances may vary between different runs (e.g., due to different temperatures or analysts). However, the shape of the standard curve should be similar to the one given in the quality assurance certificate.

Minimum requirements are as follows:

(*1*) OD at 450 nm for standard 1 higher than 0.8.

(*2*) OD values for standards should continuously decrease with higher concentrations, especially when comparing standard 1 (0 ng/mL) and standard 2 (20 ng/mL).

(*3*) An OD value for standard 1 that is much higher than the OD value stated in the certificate could be an indication of errors during pipetting or incubation.

Results and Discussion

Collaborative Study Results

After finishing the analysis, each participant sent the data to the Study Coordinator. These results are given in Table 1. After statistical analysis of the data set, three problem laboratories were identified. Further review found Laboratory F did not run the calibrators in duplicate determinations as directed. Laboratory E found no difference between calibration standards S1 and S2, and as a consequence, a high OD difference between standards S4 and S5 led to an unusual curve shape. An interview with Laboratory E also revealed technical problems during sample preparation. Laboratory K had a variation in the calibration curve that was too high, and an interview revealed the possibility of gluten contamination in the laboratory and incorrect pipetting. As a result of these deviations, all data from Laboratories E, F, and K were excluded from the statistical evaluation.

For sample 5 (naturally contaminated syrup), all values were calculated by cubic spline. Due to the fact that some OD values were below the OD values of standard 2 (10 ng/mL prolamin; corresponds to concentration of 10 mg/kg in the sample), these values were extrapolated by the software. For the gluten-free samples 1 and 4 the RIDA®SOFT Win software returned only a result of <10 mg/kg, and extrapolation led to unrealistic values. To be able to use the results of the analysis of the gluten-free samples 1 and 4 in the performance statistics, estimates of concentration values for these samples were required. For this purpose, the calibration curves were constructed by using a second-order polynomial model and used to recalculate the results for samples 1 and 4 (7). This calibration provided an estimate of concentrations for the gluten-free samples (Tables 1 and 2).

Statistical Analysis and Discussion

The remaining data of 13 laboratories are shown in Table 2 and were used to calculate the necessary statistics. Only three outlying values were identified according to AOAC INTERNATIONAL guidelines (12). These are indicated in Table 2 by the superscripts "c" (for a Cochran outlier) and "d" (for a double Grubbs' outlier). The performance statistics without outliers are shown in Table 2015.05.

From the measured overall mean concentrations of the gluten-containing samples, recovery rates were calculated.

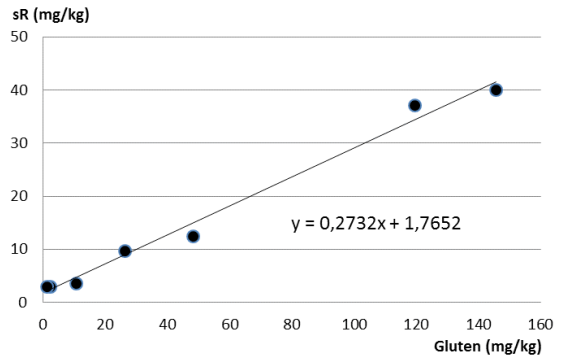

Figure 1. Plot of reproducibility (y-axis) versus the global mean observed gluten concentration for the interlaboratory study (x-axis).

The recovery values for samples 2, 3, 6, and 7 were 87, 119, 69, and 97%, respectively. The range of recoveries complies with acceptable recovery rates suggested by Abbott et al. (16) for spiked food samples, incurred samples, and/or difficult matrixes. For sample 5 (naturally contaminated starch syrup), no recovery rate could be calculated because the initial gluten content was not known. For sample 6 (sourdough spiked with 70 mg/kg), the mean recovery for all laboratories was 69%. Since the recovery for sample 7 (sourdough at 150 mg/kg) was 97%, the lower recovery could not be attributed to the matrix or the homogenization before the collaborative test. It could be speculated that a systematic error occurred during mixing the gluten-free quinoa sourdough with a rye sourdough because only minute amounts of the rye sourdough were weighed and mixed. The repeatability RSD (RSD_r) was comparable for all gluten-containing samples, ranging from 16 to 32%. This was also the case for sample 5 (naturally contaminated starch syrup), which had an average concentration of 10.6 mg/kg gluten, which was close to the LOQ specified by the manufacturer. Although the RSD_R was somewhat higher, it was limited to a maximum RSD_R of 37%. According to Abbott et al. (16), the LOD is calculated from the equation in Figure 1 at 10.6 mg/kg. The mean concentration of the blank samples was not included into this calculation since the uncertainty of this estimation is very high, and furthermore, very low gluten contaminations cannot be excluded.

Discussion

The immunochemical method for competitive gluten quantitation that was evaluated by the collaborative study described in this report is designed for the detection of the gluten content in syrups and fermented foods. In these samples, gluten is present as fragments generated by partial hydrolysis due to the action of peptidases. The method should be able to detect gluten fragments in concentrations well below 20 mg/kg gluten according to the Codex Alimentarius (1), European Union regulation 41/2009 (2), and the U.S. Food and Drug Administration (3). The assay described in this study has been shown to be more reliable for this type of samples than the sandwich version (AACCI Method 38-50.01), which is designed for quantitating nonhydrolyzed gluten (8). The analytical range of this method is estimated to be from 10.6 to 150 mg/kg.

Conclusions

The collaborative study has shown that the competitive R5 ELISA is capable of analyzing gluten fragments at concentrations starting at 10.6 up to 150 mg/kg. The competitive R5 assay enabled quantitation below and above gluten concentrations of 20 mg/kg.

The PT digest does not represent all hydrolysis processes. There are many additional factors, including temperature and time, that can affect the accuracy of the assay. Users should confirm method performance for their specific processes.

Acknowledgments

We wish to thank

Peter Koehler, Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany

Clyde Don, Foodphysica, Driel, The Netherlands

Michael Tilley, USDA-ARS, Manhattan, KS

Ulrike Immer, R-Biopharm AG, Darmstadt, Germany

Theresa Schwalb, Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany

Paul Wehling, General Mills, Minneapolis, MN

Patricia Meinhardt, R-Biopharm Inc., Washington, MO

Christian Goesswein, R-Biopharm AG, Darmstadt, Germany

Tina Dubois, R-Biopharm AG, Darmstadt, Germany

Terry Nelsen, AACCI, Minneapolis, MN

Greg Grahek, AACCI, Minneapolis, MN

for their useful contributions to this successful study.

The participation of the following laboratories in the collaborative study is gratefully acknowledged.

Petra Lutter, Nestle Research Center, Lausanne, Switzerland

Guenther Augustin, Dr. Schär S.r.l., Postal, Italy

Sandor Tömösközi, University of Technology and Economics, Budapest, Hungary

Ulrike Tamm, Eurofins, Hamburg, Germany

Tuula Sontag-Strohm, University of Helsinki, Helsinki, Finland

YlvaSjögren Bolin, National Food Administration, Uppsala, Sweden

Ulrike Immer, R-Biopharm AG, Darmstadt, Germany

Rupert Hochegger, Agentur Gesundheit Ernährungssicherheit (AGES), Wien, Austria

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Cassidy Meeks, General Mills, Golden Valley, MN

Dan Thompson, Eurofins, Metairie, LA

Christine Poirier, Health Canada, Ottawa, Canada

Janette Gelroth, AIB International, Manhattan, KS

Peter Cressey, Institute of Environmental Science and Research Ltd, Christchurch, New Zealand

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AOAC Official Method 2015.06
Minerals and Trace Elements
in Infant Formula and Adult/Pediatric Nutritional Formula
ICP/MS Method
First Action 2015

(Applicable for determination of Na, Mg, P, K, Ca, Cr, Mn, Fe, Cu, Zn, Se, and Mo in infant formula and adult/pediatric nutritional formula.)

Caution: Chemicals employed are common-use solvents and reagents, harmful if inhaled, swallowed, or absorbed through the skin. Refer to adequate manuals or Materials Safety Data Sheets (MSDSs) to ensure that the safety guidelines are applied before using chemicals. Microwave operation involves hot pressurized acid solution. Use appropriate personal protective equipment such as a laboratory coat, safety glasses, rubber gloves, and a fume hood. Dispose of all materials according to federal, state, and local regulations.

A. Principle

This method is an extension of AOAC Final Action Method **2011.19** to determine nine additional elements. Nitric acid, ISTD, and hydrogen peroxide are added to the sample in microwave vessels, and the samples are digested using preprogrammed temperature control. The addition of hydrogen peroxide helps reduce carbon and nitrous oxide levels in the digestate. The presence of carbon in the samples causes signal enhancement of Se. Therefore, to matrix match the samples, carbon in the form of methanol is added to both the standard solutions and the digestate before analysis. Ge (for 11 elements) and Te (just for Se) are used as ISTDs. Analysis is performed by ICP/MS. Polyatomic interferences with the low mass elements are reduced or eliminated by analyzing in the He collision mode using kinetic energy discrimination (KED). For Se measurements, the H_2 gas mode is preferred for increased sensitivity. Quantitation of 12 elements is achieved essentially simultaneously by comparing the analyte/ISTD response ratios in the unknown samples to a standard curve constructed from response ratios of calibration standards.

B. Apparatus

(**a**) *ICP mass spectrometer.*—With quartz spray chamber, quartz torch, Ni/Pt sample cone, Ni/Pt skimmer cone, autosampler, and printer. The ICP mass spectrometer must have collision reaction cells (CRCs). In a limited multilaboratory testing study, four different ICP/MS instrument models from three major vendors delivered equivalent performance.

(**b**) *Microwave oven.*—Commercial microwave designed for laboratory use at 0–300°C, with closed vessel system and controlled temperature ramping capability. Use manufacturers' recommended vessels. (*Caution*: microwave operation involves hot pressurized acid solution. Use appropriate face protection and laboratory clothing.)

(**c**) *Hydrogen generator (hydrogen is recommended for better Se sensitivity).*—Parker Balston (Haverhill, MA) Model H2PD-150, or equivalent. Alternatively, a high pressure cylinder (99.999% purity) may be used.

(**d**) *Magnetic stir plate.*

(**e**) *Teflon-coated magnetic stir bars.*

(**f**) *Analytical balance.*—Capable of weighing to 0.0001 g.

(**g**) *Fume hood.*

(**h**) *Common laboratory glassware/plasticware.*

(**i**) *Repipetter.*—50 mL.

(**j**) *Bottle top dispenser.*—Teflon; adjustable volume 0.5–5 mL (BDH Aristar, or equivalent).

(**k**) *Volumetric pipets.*—Class A, assorted sizes.

(**l**) *Digital pipets.*—1 mL (Rainin EDP-Plus, or equivalent).

C. Reagents

(**a**) *Multielement standard stock solution.*—NIST or NIST-traceable containing Se at 20 µg/L; Cr and Mo at 40 µg/L; Mn and Cu at 0.25 mg/L; Zn at 1 mg/L; Fe at 2.5 mg/L; Mg at 10 mg/L; P at 25 mg/L; Ca and K at 50 mg/L; and Na at 25 mg/L in 2% HNO_3 + trace HF. This stock standard solution expires on the date given by the manufacturer.

(**b**) *Multielement ISTD stock solution.*—NIST or NIST-traceable containing Ge and Te at 5 mg/L in 2% HNO_3 + trace HF. This stock standard solution expires on the date given by the manufacturer.

(**c**) *Tuning and pulse/analog* (*P/A) factor tuning stock solutions.*—NIST or NIST-traceable containing various elements at concentration levels recommended by the manufacturer. Since this ICP/MS method determines the major elements at relatively high concentrations, it is important to understand the solutions needed and the procedure to obtain high quality calibration curves in which the detector is used in both pulse counting and analog modes. A properly calibrated instrument will deliver the linearity requirements of the method; for example, that calibration residuals are <4% (*see* section F).

(**d**) *QCS.*—Standard Reference Material 1849a (NIST) milk-based hybrid infant/adult nutritional powder with certified values for Ca, Cu, Cr, Fe, Mg, Mn, Mo, P, K, Se, Na, and Zn. Supplied as a unit of 10 packets each containing approximately 10 g material. This is the recommended control material for this analysis, but other suitable SRMs could be substituted.

(**e**) *Methanol.*—99.99%, analytical reagent grade.

(**f**) *Nitric acid.*—Concentrated, ultrapure reagent grade (J.T. Baker, Phillipsburg, NJ; Ultrex II or equivalent).

(**g**) *Nitric acid.*—Concentrated, trace metal grade (BDH Aristar Plus or equivalent).

(**h**) *Hydrogen peroxide, 30%.*—ACS reagent grade.

(**i**) *Laboratory water.*—Millipore treated, 18 MΩ cm, or equivalent.

(**j**) *Tergitol®.*—Type 15-S-9, Sigma or equivalent surfactant.

(**k**) *Argon gas.*—≥99.996% purity.

(**l**) *Helium gas.*—≥99.9999% purity.

(**m**) *Hydrogen gas*.—≥99.9995% purity, for Se analyses (recommended).

D. Preparation of Standards and Solutions

(**a**) *Tergitol solution (approximately 5%).*—Add about 700 mL laboratory water to a 1 L plastic bottle containing a Teflon-coated stirring bar. Place the bottle on a magnetic stirrer and begin stirring at a moderate speed. Slowly add 50 mL Tergitol from a graduated cylinder. When the Tergitol is dissolved, fill the bottle to approximately 1000 mL with laboratory water. Transfer to a 1 L plastic bottle fitted with a Teflon-constructed dispenser with adjustable volume from 0.5 to 5 mL. This solution is added to the autosampler rinse solution to minimize residue buildup in the spray chamber. It does not otherwise affect the analysis. Expiration: 6 months; store at room temperature.

(**b**) *Nitric acid rinse solution for autosampler rinse port, 2%, with 2% Tergitol.*—Mix 20 mL concentrated nitric acid (ultrapure reagent grade) with 20 mL Tergitol solution (**a**) and laboratory water to prepare a total volume of 1000 mL. Expiration: 3 months; store at room temperature.

(**c**) *P/A factor tuning working solution.*—Dilute and/or combine P/A factor tuning stock solutions (or equivalent) to manufacturer's recommended dilution level with laboratory water for use with the instrument. Expiration: 6 months; store at room temperature.

(**d**) *Calibration blank (Cal Blk) and preparation blank (PB) solution.*—Add approximately 15 mL laboratory water to a 50 mL volumetric flask. Dispense (using bottle dispenser or pipet) 5 mL nitric acid (ultrapure reagent grade) into the same volumetric flask. Pipet (using digital pipet) 0.500 mL ISTD stock and 0.500 mL methanol into the flask. Dilute to volume with laboratory water. This solution serves as both the Cal Blk and PB. The Cal Blk is used as the initial calibration point, while the PB is used as a QCS (*see* below). Use the same lots of reagent for samples. Expiration: 2 days; store at room temperature.

(**e**) *Calibration standard solution set.*—Prepare Cal Blk, Cal Std 1, Cal Std 2, Cal Std 3, and Cal Std 4 standard solutions by pipetting (with Class A glass pipet) 0.00, 1.00, 5.00, 20.00, and 40.00 mL, respectively, of the multielement standard stock solution into separate 50 mL volumetric flasks or sample tubes. Add 0.500 mL ISTD stock (using Class A pipet or digital pipet), 5 mL (using repipetter or Teflon bottle dispenser) nitric acid (ultrapure reagent grade), and 0.500 mL methanol to each flask. Fill the flasks to volume with laboratory water. Expiration: 2 days; store at room temperature. The analyte and ISTD concentrations in the calibration standard solutions are shown in Table **2015.06A**.

E. Sample Preparation

(**a**) Prepare samples in duplicate. In sample vessels, weigh test portions to the nearest 0.0001 g. For liquid products, the test portion size is 1.0 g. For powdered products, the test portion size is net 0.20 g of a powder sample, which should be taken from a 10% (w/w) reconstitution in warm (60°C) water (i.e., 2.0 g of the 10% reconstitution). Add 0.500 mL ISTD stock using a calibrated digital pipet, 5 mL nitric acid (ultrapure reagent grade), and 2 mL 10% hydrogen peroxide. (*Note*: the PB/Cal Blk solution prepared with the standards is the correct sample blank for this method. Specifically, do not microwave digest the sample blank, which can subject the blank to contamination. Also note that the digital pipet used for the addition of ISTD solution must be calibrated at point of use to ensure that it delivers a volume of 0.500 L with an accuracy better than 0.8% and precision better than 0.2% RSD).

(**b**) Seal the vessels, and place into microwave oven. Execute a heating program equivalent to that shown in Table **2015.06B**, suitable for total digestion of the sample.

(**c**) After digestion, place vessels in a fume hood. Unscrew the cap/venting nut slowly to gradually release the pressure. Then, completely remove the cap.

(**d**) Add approximately 20 mL laboratory water to the contents of the vessel, swirl to mix, and transfer contents to a 50 mL sample vial. Add 0.5 mL methanol to the sample vial and dilute to approximately 50 mL with laboratory water. Shake briefly. The transfer or the final volume does not need to be quantitative because ISTDs were added prior to digestion; therefore, the analyte/ISTD ratios will be constant.

F. Determination

(**a**) Using the appropriate tuning solutions, tune the instrument for optimal sensitivity in the KED mode and/or reaction mode according to the instrument design. Also, tune the instrument to find the P/A calibration factors that are needed for those calibration curves that will extend above roughly 100 µg/L (depends on instrument type). Table **2015.06C** summarizes typical instrument parameters for analysis.

(**b**) Analyze test solutions using an ICP/MS instrument standardized with the indicated standard solutions (Table **2015.06A**). Ge is used as the ISTD for the 11 elements not including Se. Those 11 elements are determined in the He collision mode, employing KED. Te must be used as the ISTD for Se determinations, and we recommend that Se be determined in H_2 mode, i.e., reaction mode. Analyze Cal Std 3, or other suitable QC solution, every 10 test portions to monitor for instrument drift and linearity (result must be within 4% of the standard's nominal concentration). The inclusion of a PB (run as a sample; its measured concentration must be <1/2 of the lowest calibration standard), a duplicate sample (relative difference within 10% for Cr, 7% for Se, and 5% for all other elements), and known reference materials serving as control samples (recovery check within control or certified limits) are mandatory for good method performance. If any of these QC checks fails, results should be considered invalid.

(**c**) The order of analysis should be calibration standards, followed by rinse, blank check (PB run as a sample), check standard, control sample, sample, sample duplicate (up to 10 samples), and finally a repeated check standard.

G. Calculations

Sample concentrations in ng/g are automatically calculated by the software using a nonweighted least-squares linear regression calibration analysis to produce a best-fit line:

$$Y = ax + \text{blank}$$

Note that for the Agilent software used in this work, the sample blank is identical to the Cal Blk and is essentially zero because high purity reagents are used.

The analyte concentration in the sample was then calculated:

$$x = \frac{y - blank}{a} \times DF$$

where x = analyte concentration (ng/g); y = analyte to ISTD intensity ratio, which is the measured count of each analyte's standard solution data point in the calibration curve divided by the counts of the ISTD at the same level; similarly, the blank = analyte to ISTD intensity ratio, which is the measured count of the blank standard solution data point in the calibration curve divided by the counts of the ISTD at the same level as the blank standard solution; a = slope of the calibration curve (mL/ng); and DF = volume of the sample solution (mL) divided by sample weight (g).

H. Method Validation

This method has undergone a thorough SLV using AOAC guidelines to probe its linearity, LOQ, specificity, precision, accuracy, and ruggedness/robustness. Accuracy has also been affirmed by comparison to ICP-atomic emission spectrometry (AES) results generated in the authors' own laboratory. In addition, reproducibility was estimated during a limited multilaboratory testing (MLT) study employing six laboratories and four different ICP/MS instruments. Both the SLV and MLT results are summarized in a concurrent publication.

References: *J. AOAC Int*. (future issue)

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DOI: 10.5740/jaoac.int.11-0441

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J. AOAC Int. **98**, 1042(2015)

Posted: October 1, 2015

Table 2015.06A. Concentrations of standards and ISTD in calibration standard solutions, and corresponding practical LOQ (PLOQ)

	Na	Mg	P	K	Ca	Cr	Mn	Fe	Cu	Zn	Se	Mo
	mg/L	mg/L	mg/L	mg/L	mg/L	µg/L	mg/L	mg/L	mg/L	mg/L	µg/L	µg/L
Cal Blk	0	0	0	0	0	0	0	0	0	0	0	0
Cal Std 1[a]	0.500	0.200	0.500	1.00	1.00	0.800	0.00500	0.0500	0.00500	0.0200	0.400	0.800
Cal Std 2	2.50	1.00	2.50	5.00	5.00	4.00	0.0250	0.250	0.0250	0.100	2.00	4.00
Cal Std 3	10.0	4.00	10.0	20.0	20.0	16.0	0.100	1.00	0.100	0.400	8.00	16.0
Cal Std 4	20.0	8.00	20.0	40.0	40.0	32.0	0.200	2.00	0.200	0.800	16.0	32.0
ISTD (at 50 µg/L)	Ge	Ge	Ge	Ge	Ge	Ge	Ge	Ge	Ge	Ge	Te	Ge
PLOQ	0.25	0.10	0.25	0.50	0.50	0.40	0.0025	0.025	0.0025	0.010	0.20	0.40

[a] Calibration standard.

Table 2015.06B. Microwave operating parameters: Stages 1 and 2 are operated sequentially, without removing vessels from the oven

	Stage 1 sample digestion	
1	Power	100% (1600 W)
2	Ramp to temperature	20 min
3	Hold time	20 min
4	Temperature	180°C
5	Cool down	20 min
	Stage 2 sample digestion	
1	Power	100% (1600 W)
2	Ramp to temperature	20 min
3	Hold time	20 min
4	Temperature	200°C
5	Cool down	20 min
	Total	2 h

Table 2015.06C. Typical ICP/MS parameters for Agilent 7700x

RF power, W[a]	1600
RF matching, V	1.8
Sampling depth, mm	9
Extract 1 lens, V	0
Carrier gas, L/min	0.9
Make-up gas, L/min	0.2
Nebulizer (glass concentric)	MicroMist
Spray chamber temperature, °C	2
Interface cones	Ni
He cell gas flow rate, mL/min	4.5
H_2 cell gas flow rate, mL/min	4.2
Nebulizer pump rate, rps	0.1 (0.5 mL/min)
Peristaltic pump tubing	White/white, 1.02 mm id
Drain tubing	Blue/yellow, 1.52 mm id

[a] RF = Radio frequency.

INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Minerals and Trace Elements in Infant Formula and Adult/Pediatric Nutritional Formula by Inductively Coupled Plasma/Mass Spectrometry—A Performance Evaluation: Single-Laboratory Validation, First Action 2015.06

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A method for determination of 12 minerals and trace elements (Na, Mg, P, K, Ca, Cr, Mn, Fe, Cu, Zn, Se, and Mo) in infant formula and adult/pediatric nutritional formula was developed and evaluated in a single-laboratory validation. Some additional reproducibility data were obtained from a small interlaboratory study. The method involves microwave digestion of the sample followed by inductively coupled plasma/MS and uses Ge and Te as internal standards. The method is an extension of *Official Method*SM 2011.19 and was compared to AOAC *Standard Method Performance Requirements* (SMPRs®) 2011.009 and 2014.004 developed by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). Repeatability precision for the 12 elements in 11 SPIFAN matrixes and National Institute of Standards and Technology Standard Reference Material (SRM) 1849a was <5%, meeting the SMPR criterion for repeatability. Intermediate reproducibility (8 days, two analysts, two instruments) in the 11 SPIFAN matrixes was <5% for nine (Na, Mg, P, K, Mn, Fe, Cu, Zn, Se) of the 12 elements in all 11 matrixes. The mean reproducibility across 6–7 laboratories and seven SPIFAN matrixes ranged from 2.5% for Cu to 7.1% for P. Recovery from spiked matrixes varied from 90.1 to 109%, and accuracy of determination using SRM 1849a ranged from 96.2 to 107.7%, meeting the requirement of 90–110% recovery/accuracy.

In response to a need for reference methods for dispute resolution, the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) developed *Standard Method Performance Requirements* (SMPRs®) for *Official Methods of Analysis* for the ultratrace elements Cr, Mo, and Se (AOAC SMPR 2011.009; 1) and for the major/trace elements Ca, Mg, P, K, Na, Cu, Fe, Mn, and Zn (AOAC SMPR 2014.004; 2) in infant formula and adult/pediatric nutritional formula. AOAC *Official Method*SM **2011.19** (3) for determination of Cr, Mo, and Se, previously validated and shown to conform to the requirements of SMPR 2011.009, was expanded to include the minerals and trace elements contained in SMPR 2014.004. The method includes addition of internal standards (ISTDs) prior to microwave digestion of the sample in the presence of nitric acid and hydrogen peroxide. The diluted digestate is analyzed by inductively coupled plasma (ICP)/MS, and response ratios of analyte:ISTD in unknown samples are compared to external calibration curves to generate a result for each analyte.

AOAC SMPR 2011.009 (1) provides the performance criteria for Cr, Mo, and Se, and SMPR 2014.004 (2) provides the performance criteria for Ca, Cu, Fe, Mg, Mn, P, K, Na, and Zn. The criteria are summarized in Table 1. This manuscript reports the results of the single-laboratory validation (SLV) and some limited reproducibility data for the ICP/MS method for 12 minerals and trace elements and comparison to the acceptance criteria. Based on the data presented, the AOAC Expert Review Panel on SPIFAN Nutrient Methods granted the method First Action status for the nine elements in March 2015. Note that similar data for just Cr, Mo, and Se were presented in a prior publication (4), but that SLV was conducted on a different set of matrixes.

The SLV was conducted in accordance with the guidelines recommended by SPIFAN (5) and included determinations of specificity, linearity, LOQ, repeatability precision, and recovery/accuracy in 11 matrixes identified by SPIFAN. The matrixes cover a variety of nutritional formulations, including powders and ready-to-feed (RTF) liquids made from milk, soy, whey, hydrolyzed protein, and amino acids, with and without intact protein. This SLV was performed by two analysts using two Agilent (Santa Clara, CA) ICP/MS instruments, one a 7500cx and the other a 7700x. The microwave oven was a CEM Corp. (Matthews, NC) MARS 5 with MARSXpress™ vessels.

Submitted for publication June 1, 2015.
This method was approved by the Expert Review Panel for Infant Formula and Adult Nutritionals as First Action.
The Expert Review Panel for Infant Formula and Adult Nutritionals invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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DOI: 10.5740/jaoacint.15-142

Table 1. Summary of SLV acceptance criteria from SMPR 2011.009 and SMPR 2014.004

Parameter	SMPR 2014.004										SMPR 2011.009		
	Mn	Cu	Fe	Zn	Mg	P	Na	Ca	K	Cr	Mo	Se
Analytical range[a,b]	0.001–1.0	0.001–1.2	0.01–20	0.1–18	3–110	15–800	10–850	20–1280	10–2000	20–1600	20–1000	10–500
LOD[a,b]	NA[c]	NA	NA	NA	NA	NA	NA	NA	NA	7	7	4
LOQ[a,b]	0.001	0.001	0.01	0.1	3	15	10	20	10	20	20	10
Repeatability	≤5% at >0.005[b] ≤8% at <0.005[b]						≤5%					
Recovery	90–110% at >0.005[b] 80–115% at <0.005[b]	90–110% of mean spiked recovery over the range of the assay										
Reproducibility (over the analytical range)	≤10%	≤10%	≤10%	≤10%	≤10%	≤8%	≤8%	≤8%	≤8%	≤15%	≤15%	≤15%

[a] Concentrations apply to a) RTF liquids "as-is"; b) reconstituted powders (25 g into 200 g of water); and c) liquid concentrated diluted 1:1 by weight.

[b] mg/100 g reconstituted final product for Mn, Cu, Fe, Zn, Mg, P, Na, Ca, and K; µg/100 g for Cr, Mo, Se.

[c] NA = Not applicable.

Specificity and linearity studies were conducted with pure analyte (no matrix). For the specificity studies, individual standards of each analyte were prepared at 50 mg/L. For linearity, standards were prepared at nine concentrations of each analyte, spanning a range from 50% of the lowest calibration standard to 50% above the highest calibration standard. For precision studies, the 11 SPIFAN matrixes (infant powder, milk; infant powder, soy; infant powder, milk partially hydrolyzed; infant powder, soy partially hydrolyzed; adult powder, low fat; adult powder, milk; pediatric formula powder; infant elemental powder; infant RTF, milk; adult RTF, high protein; and adult RTF, high fat) were tested for all 12 elements on 8 days in duplicate each day, by two analysts using two Agilent ICP/MS units. For accuracy/recovery studies, each SPIFAN matrix was spiked with various concentrations of each analyte and analyzed by the method in triplicate over 3 days. Finally, Standard Reference Material (SRM) 1849a [National Institute of Standards and Technologies (NIST), Gaitherburg, MD] was used to verify accuracy. The SRM is a milk-based hybrid infant/adult nutritional powder with certified values for Ca, Cu, Cr, Fe, Mg, Mn, Mo, P, K, Se, Na, and Zn and was included as a QC sample (QCS) in each run of the method.

AOAC Official Method 2015.06
Minerals and Trace Elements in Infant Formula and Adult/Pediatric Nutritional Formula
ICP/MS Method
First Action 2015

(Applicable for determination of Na, Mg, P, K, Ca, Cr, Mn, Fe, Cu, Zn, Se, and Mo in infant formula and adult/pediatric nutritional formula.)

Caution: Chemicals employed are common-use solvents and reagents, harmful if inhaled, swallowed, or absorbed through the skin. Refer to adequate manuals or Materials Safety Data Sheets (MSDSs) to ensure that the safety guidelines are applied before using chemicals. Microwave operation involves hot pressurized acid solution. Use appropriate personal protective equipment such as a laboratory coat, safety glasses, rubber gloves, and a fume hood. Dispose of all materials according to federal, state, and local regulations.

A. Principle

This method is an extension of AOAC Final Action Method **2011.19** to determine nine additional elements. Nitric acid, ISTD, and hydrogen peroxide are added to the sample in microwave vessels, and the samples are digested using preprogrammed temperature control. The addition of hydrogen peroxide helps reduce carbon and nitrous oxide levels in the digestate. The presence of carbon in the samples causes signal enhancement of Se. Therefore, to matrix match the samples, carbon in the form of methanol is added to both the standard solutions and the digestate before analysis. Ge (for 11 elements) and Te (just for Se) are used as ISTDs. Analysis is performed by ICP/MS. Polyatomic interferences with the low mass elements are reduced or eliminated by analyzing in the He collision mode using kinetic energy discrimination (KED). For Se measurements, the H_2 gas mode is preferred for increased sensitivity. Quantitation of 12 elements

is achieved essentially simultaneously by comparing the analyte/ISTD response ratios in the unknown samples to a standard curve constructed from response ratios of calibration standards.

B. Apparatus

(a) *ICP mass spectrometer.*—With quartz spray chamber, quartz torch, Ni/Pt sample cone, Ni/Pt skimmer cone, autosampler, and printer. The ICP mass spectrometer must have collision reaction cells (CRCs). In a limited multilaboratory testing study, four different ICP/MS instrument models from three major vendors delivered equivalent performance.

(b) *Microwave oven.*—Commercial microwave designed for laboratory use at 0–300°C, with closed vessel system and controlled temperature ramping capability. Use manufacturers' recommended vessels. (*Caution*: Microwave operation involves hot pressurized acid solution. Use appropriate face protection and laboratory clothing.)

(c) *Hydrogen generator (hydrogen is recommended for better Se sensitivity).*—Parker Balston (Haverhill, MA) Model H2PD-150, or equivalent. Alternatively, a high pressure cylinder (99.999% purity) may be used.

(d) *Magnetic stir plate.*

(e) *Teflon-coated magnetic stir bars.*

(f) *Analytical balance.*—Capable of weighing to 0.0001 g.

(g) *Fume hood.*

(h) *Common laboratory glassware/plasticware.*

(i) *Repipetter.*—50 mL.

(j) *Bottle top dispenser.*—Teflon; adjustable volume 0.5–5 mL (BDH Aristar, Radnor, PA), or equivalent.

(k) *Volumetric pipets.*—Class A, assorted sizes.

(l) *Digital pipets.*—1 mL (Rainin EDP-Plus, Oakland, CA) or equivalent.

C. Reagents

(a) *Multielement standard stock solution.*—NIST or NIST-traceable containing Se at 20 µg/L; Cr and Mo at 40 µg/L; Mn and Cu at 0.25 mg/L; Zn at 1 mg/L; Fe at 2.5 mg/L; Mg at 10 mg/L; P at 25 mg/L; Ca and K at 50 mg/L; and Na at 25 mg/L in 2% HNO_3 + trace hydrofluoric acid (HF). This stock standard solution expires on the date given by the manufacturer.

(b) *Multielement ISTD stock solution.*—NIST or NIST-traceable containing Ge and Te at 5 mg/L in 2% HNO_3 + trace HF. This stock standard solution expires on the date given by the manufacturer.

(c) *Tuning and pulse/analog (P/A) factor tuning stock solutions (High-Purity Standards, Charleston, SC, or equivalent).*—NIST or NIST-traceable containing various elements at concentration levels recommended by the manufacturer. Because this ICP/MS method determines the major elements at relatively high concentrations, it is important to understand the solutions needed and the procedure to obtain high quality calibration curves in which the detector is used in both pulse counting and analog modes. A properly calibrated instrument will deliver the linearity requirements of the method, for example, that calibration residuals are <4% (*see* section **F**).

(d) *QCS.*—Standard Reference Material (SRM) 1849a (NIST) milk-based hybrid infant/adult nutritional powder with certified values for Ca, Cu, Cr, Fe, Mg, Mn, Mo, P, K, Se, Na, and Zn. Supplied as a unit of 10 packets each containing approximately 10 g material. This is the recommended control material for this analysis, but other suitable SRMs could be substituted.

(e) *Methanol.*—99.99%, analytical reagent grade.

(f) *Nitric acid.*—Concentrated, ultrapure reagent grade (J.T. Baker, Phillipsburg, NJ; Ultrex II or equivalent).

(g) *Nitric acid.*—Concentrated (65–70%, w/v), trace metal grade (BDH Aristar Plus, West Chester, PA, or equivalent).

(h) *Hydrogen peroxide, 30%.*—ACS reagent grade.

(i) *Laboratory water.*—Millipore treated, 18 MΩ cm, or equivalent.

(j) *Tergitol®.*—Type 15-S-9, Sigma or equivalent surfactant.

(k) *Argon gas.*—≥99.996% purity.

(l) *Helium gas.*—≥99.9999% purity.

(m) *Hydrogen gas.*—≥99.9995% purity, for Se analyses (recommended).

D. Preparation of Standards and Solutions

(a) *Tergitol solution (approximately 5% v/v).*—Add about 700 mL laboratory water to a 1 L plastic bottle containing a Teflon-coated stirring bar. Place the bottle on a magnetic stirrer and begin stirring at a moderate speed. Slowly add 50 mL Tergitol from a graduated cylinder. When the Tergitol is dissolved, fill the bottle to approximately 1000 mL with laboratory water. Transfer to a 1 L plastic bottle fitted with a Teflon-constructed dispenser with adjustable volume from 0.5 to 5 mL. This solution is added to the autosampler rinse solution to minimize residue buildup in the spray chamber. It does not otherwise affect the analysis. Expiration: 6 months; store at room temperature.

(b) *Nitric acid rinse solution (2% v/v) for autosampler rinse port with Tergitol added.*—Mix 20 mL concentrated nitric

Table 2015.06A. Concentrations of standards and ISTD in calibration standard solutions, and corresponding practical LOQ (PLOQ)

	Na, mg/L	Mg, mg/L	P, mg/L	K, mg/L	Ca, mg/L	Cr, µg/L	Mn, mg/L	Fe, mg/L	Cu, mg/L	Zn, mg/L	Se, µg/L	Mo, µg/L
Cal Blk	0	0	0	0	0	0	0	0	0	0	0	0
Cal Std 1[a]	0.500	0.200	0.500	1.00	1.00	0.800	0.00500	0.0500	0.00500	0.0200	0.400	0.800
Cal Std 2	2.50	1.00	2.50	5.00	5.00	4.00	0.0250	0.250	0.0250	0.100	2.00	4.00
Cal Std 3	10.0	4.00	10.0	20.0	20.0	16.0	0.100	1.00	0.100	0.400	8.00	16.0
Cal Std 4	20.0	8.00	20.0	40.0	40.0	32.0	0.200	2.00	0.200	0.800	16.0	32.0
ISTD (at 50 µg/L)	Ge	Ge	Ge	Ge	Ge	Ge	Ge	Ge	Ge	Ge	Te	Ge
PLOQ	0.25	0.10	0.25	0.50	0.50	0.40	0.0025	0.025	0.0025	0.010	0.20	0.40

[a] Calibration standard.

Table 2015.06B. Microwave operating parameters: Stages 1 and 2 are operated sequentially, without removing vessels from the oven

	Stage 1 sample digestion	
1	Power	100% (1600 W)
2	Ramp to temp., min	20
3	Hold time	20
4	Temp., °C	180
5	Cool down, min	20
	Stage 2 sample digestion	
1	Power	100% (1600 W)
2	Ramp to temp., min	20
3	Hold time, min	20
4	Temp., °C	200
5	Cool down, min	20
Total, h		2

acid (ultrapure reagent grade) with 20 mL Tergitol solution (**a**) and laboratory water to prepare a total volume of 1000 mL. Expiration: 3 months; store at room temperature.

(c) *P/A factor tuning working solution.*—Dilute and/or combine P/A factor tuning stock solutions (or equivalent) to manufacturer's recommended dilution level with laboratory water for use with the instrument. Expiration: 6 months; store at room temperature.

(d) *Calibration blank (Cal Blk) and preparation blank (PB) solution.*—Add approximately 15 mL laboratory water to a 50 mL volumetric flask. Dispense (using bottle dispenser or pipet) 5 mL nitric acid (ultrapure reagent grade) into the same volumetric flask. Pipette (using digital pipet) 0.500 mL ISTD stock and 0.500 mL methanol into the flask. Dilute to volume with laboratory water. This solution serves as both the Cal Blk and PB. The Cal Blk is used as the initial calibration point, while the PB is used as a QCS (*see* below). Use the same lots of reagent for samples. Expiration: 2 days; store at room temperature.

(e) *Calibration standard solution set.*—Prepare Cal Blk, Cal Std 1, Cal Std 2, Cal Std 3, and Cal Std 4 standard solutions by pipetting (with Class A glass pipet) 0.00, 1.00, 5.00, 20.00, and 40.00 mL, respectively, of the multielement standard stock solution into separate 50 mL volumetric flasks or sample tubes. Add 0.500 mL ISTD stock (using Class A pipet or digital pipet), 5 mL (using repipetter or Teflon bottle dispenser) nitric acid (ultrapure reagent grade), and 0.500 mL methanol to each flask. Fill the flasks to volume with laboratory water. Expiration: 2 days; store at room temperature. The analyte and ISTD concentrations in the calibration standard solutions are shown in Table **2015.06A**.

E. Sample Preparation

(a) Prepare samples in duplicate. In sample vessels, weigh test portions to the nearest 0.0001 g. For liquid products, the test portion size is 1.0 g. For powdered products, the test portion size is net 0.20 g of a powder sample, which should be taken from a 10% (w/w) reconstitution in warm (60°C) water (i.e., 2.0 g of the 10% reconstitution). Add 0.500 mL ISTD stock using a calibrated digital pipet, 5 mL nitric acid (ultrapure reagent grade), and 2 mL 10% hydrogen peroxide. (*Note*: the PB/Cal Blk solution prepared with the standards is the correct sample blank for this method. Specifically, do not microwave digest the sample blank, which can subject the blank to contamination. Also note that the digital pipet used for the addition of ISTD solution must be calibrated at point of use to ensure that it delivers a nominal volume of 0.500 mL within a tolerance of ±0.8% and precision better than 0.2% RSD).

(b) Seal the vessels, and place into microwave oven. Execute a heating program equivalent to that shown in Table **2015.06B**, suitable for total digestion of the sample.

(c) After digestion, place vessels in a fume hood. Unscrew the cap/venting nut slowly to gradually release the pressure. Then, completely remove the cap.

(d) Add approximately 20 mL laboratory water to the contents of the vessel, swirl to mix, and transfer contents to a 50 mL sample vial. Add 0.5 mL methanol to the sample vial and dilute to approximately 50 mL with laboratory water. Shake briefly. The transfer or the final volume does not need to be quantitative because ISTDs were added prior to digestion; therefore, the analyte/ISTD ratios will be constant.

F. Determination

(a) Using the appropriate tuning solutions, tune the instrument for optimal sensitivity in the KED mode and/or reaction mode according to the instrument design. Also, tune the instrument to find the P/A calibration factors that are needed for those calibration curves that will extend above roughly 100 µg/L (depends on instrument type). Table **2015.06C** summarizes typical instrument parameters for analysis.

(b) Analyze test solutions using an ICP/MS instrument standardized with the indicated standard solutions (Table **2015.06A**). Ge is used as the ISTD for the 11 elements not including Se. Those 11 elements are determined in the He collision mode, using KED. Te must be used as the ISTD for Se determinations, and we recommend that Se be determined in H_2 mode, i.e., reaction mode. Analyze Cal Std 3, or other suitable QC solution, every 10 test portions to monitor for instrument drift and linearity (result must be within 4% of

Table 2015.06C. Typical ICP/MS parameters for Agilent 7700x

RF power, W[a]	1600
RF matching, V	1.8
Sampling depth, mm	9
Extract 1 lens, V	0
Carrier gas, L/min	0.9
Make-up gas, L/min	0.2
Nebulizer (glass concentric)	MicroMist
Spray chamber temp., °C	2
Interface cones	Ni
He cell gas flow rate, mL/min	4.5
H_2 cell gas flow rate, mL/min	4.2
Nebulizer pump rate, rps	0.1 (0.5 mL/min)
Peristaltic pump tubing	White/white, 1.02 mm id
Drain tubing	Blue/yellow, 1.52 mm id

[a] RF = Radio frequency.

the standard's nominal concentration). The inclusion of a PB (run as a sample; its measured concentration must be <1/2 of the lowest calibration standard), a duplicate sample (relative difference within 10% for Cr, 7% for Se, and 5% for all other elements), and known reference materials serving as control samples (recovery check within control or certified limits) are mandatory for good method performance. If any of these QC checks fails, results should be considered invalid.

(c) The order of analysis should be calibration standards, followed by rinse, blank check (PB run as a sample), check standard, control sample, sample, sample duplicate (up to 10 samples), and finally a repeated check standard.

G. Calculations

Sample concentrations in ng/g are automatically calculated by the software using a nonweighted least-squares linear regression calibration analysis to produce a best-fit line:

$$Y = ax + \text{blank}$$

Note that for the Agilent software used in this work, the sample blank is identical to the Cal Blk and is essentially zero because high purity reagents are used.

The analyte concentration in the sample is then calculated:

$$x = \frac{y - blank}{a} \times DF$$

where x = analyte concentration (ng/g); y = analyte to ISTD intensity ratio, which is the measured count of each analyte's standard solution data point in the calibration curve divided by the counts of the ISTD at the same level; similarly, the blank = analyte to ISTD intensity ratio, which is the measured count of the blank standard solution data point in the calibration curve divided by the counts of the ISTD at the same level as the blank standard solution; a = slope of the calibration curve (mL/ng); and DF = volume of the sample solution (mL) divided by sample weight (g).

H. Method Validation

This method has undergone a thorough single-laboratory validation (SLV) using AOAC guidelines to probe its linearity, LOQ, specificity, precision, accuracy, and ruggedness/robustness. Accuracy has also been affirmed by comparison to ICP-atomic emission spectrometry (AES) results generated in the authors' own laboratory. In addition, reproducibility was estimated during a limited multilaboratory testing (MLT) study employing six laboratories and four different ICP/MS instruments. Both the SLV and MLT results are summarized in a concurrent publication (6).

Results and Discussion

Specificity

The specificity of the method was determined using a single element standard at 50 mg/L for each analyte and checking for apparent signal from the other analytes. None of the standards produced a response above the PLOQ for any of the other 11 analytes (data not shown), demonstrating that each response is specific for that analyte. The ISTDs were not tested since they are used at a low concentration of 50 µg/L.

Linearity

Linearity was demonstrated by analyzing various independent standards (made from the same stock) as samples against the normal calibration curve. Linearity standards at nine concentrations of each analyte spanning the range from 50% of the lowest calibration standard to 50% above the highest calibration standard were analyzed twice on each of 3 days using freshly made standards each day. The means of all six analyses are reported in Table 2. At the lowest level, 50% of the lowest calibration standard, all analytes demonstrated acceptable agreement (95–105%, with rounding) with the nominal value. Therefore, 50% of the lowest calibration standard concentration is set as the PLOQ. Overall, the recoveries varied from 91 to 107%, and RSDs varied from 0.3 to 9.3%. The recoveries were nearly all within a desired 95–105% range, though there are no specific criteria in the SMPR for linearity. The only elements that presented any linearity issues were P and Fe, which were routinely under-recovered (P) or over-recovered (Fe) by about 5–6% across the calibration curve. Possibly, the linearity could be improved by adjusting some factors for the analysis of these elements, as they both have relatively low mass with significant background interferences that must be handled by the CRC. In practice, no accuracy issues were observed except for some apparent bias in P results relative to SRM 1849a (*see* below). Typical correlation coefficients were 0.9995 or better for all analytes.

LOQ

The PLOQ values from the linearity experiment were converted from a solution concentration (mg/L) to a weight basis (mg/100 g for a typical dilution of 1.0 g RTF to 50 mL) and compared to the SMPR (*see* Table 3). The PLOQs meet the SMPR for all elements except Fe, Cu, and Mn. In these cases, the test portion size could be increased to 2–3 g RTF to improve the PLOQ 2–3-fold lower. The lowest concentrations of Mn, Cu, and Fe found in the SPIFAN matrixes were 150 ng/g (0.015 mg/100 g), 580 ng/g (0.058 mg/100 g), and 14 000 ng/g (1.4 mg/100 g), respectively, all in the SPIFAN control milk. SMPR for LOQ for Mn, Cu, and Fe are 0.001, 0.001, and 0.01 mg/100 g, respectively, at least 10-fold lower than observed values.

Precision

SPIFAN matrixes were tested on 8 days (including two analysts and two instruments) in duplicate, and the results are summarized in Table 4. The SMPRs require RSD_r to be ≤5% in all 11 matrixes. All analytes in all matrixes meet this criterion for the within-day duplicates (data not shown), typically in the 1–2% range. This requirement is built into the method due to the criterion that duplicate results must agree to within 5%. When considering intermediate reproducibility precision (among days/analysts/instruments, but in a single laboratory), of the 12 elements and 11 matrixes, there are 11 instances of RSD_{iR} >5%. Ten of these are for the ultratrace elements, Mo and Cr, and there is one instance for Ca in Adult RTF with high fat. The Adult RTF with high fat matrix has since been shown to be unstable and perhaps

Table 2. Linearity determination—average of duplicate results from 3 separate days (n = 3). These determinations were performed after calibration with the standards listed in Table 3

Element	Parameter									
Na[a]	Expected value, mg/L	0.25	0.50	0.75	1.25	1.5	5.0	10	15	30
	Overall recovery, %	102.1	104.7	104.0	105.7	105.2	102.4	101.5	100.5	100.1
	Overall RSD, %	5.3	2.9	2.1	1.2	1.9	0.6	0.3	1.4	1.4
Mg[a]	Expected value, mg/L	0.10	0.20	0.30	0.50	0.60	2.0	4.0	6.0	12.0
	Overall recovery, %	98.9	101.8	103.7	102.9	101.3	100.5	101.1	99.4	99.3
	Overall RSD, %	4.6	1.9	2.5	1.8	0.6	0.7	1.6	1.1	1.1
P[a]	Expected value, mg/L	0.25	0.50	0.75	1.25	1.5	5.0	10.0	15.0	30.0
	Overall recovery, %	94.8	97.6	93.0	93.4	91.1	93.9	96.1	97.3	93.8
	Overall RSD, %	5.9	1.2	7.5	1.3	9.3	8.1	7.8	0.5	8.0
K[a]	Expected value, mg/L	0.50	1.0	1.5	2.5	3.0	10.0	20.0	30.0	60.0
	Overall recovery, %	98.2	100.3	101.5	103.1	101.8	102.9	102.2	101.4	100.9
	Overall RSD, %	6.9	2.6	3.1	3.7	2.3	3.2	3.2	4.2	4.0
Ca[a]	Expected value, mg/L	0.50	1.0	1.5	2.5	3.0	10.0	20.0	30.0	60.0
	Overall recovery, %	97.8	99.9	100.5	102.1	100.7	100.9	102.1	100.6	101.1
	Overall RSD, %	5.5	2.5	1.8	3.0	1.1	2.7	3.2	3.7	3.0
Cr[a]	Expected value, µg/L	0.40	0.80	1.2	2.0	2.4	8.0	16.0	24.0	48.0
	Overall recovery, %	100.2	100.1	102.0	101.5	103.0	101.8	101.5	100.9	98.9
	Overall RSD, %	7.2	5.4	0.5	1.6	1.8	1.0	0.6	1.2	2.7
Mn[a]	Expected value, mg/L	0.0025	0.005	0.0075	0.0125	0.015	0.050	0.10	0.20	0.30
	Overall recovery, %	99.9	101.1	101.7	102.3	101.9	101.9	102.4	101.4	99.6
	Overall RSD, %	5.8	3.1	1.2	1.2	0.8	1.5	1.6	1.2	1.2
Fe[a]	Expected value, mg/L	0.025	0.050	0.075	0.125	0.15	0.50	1.0	1.5	3.0
	Overall recovery, %	105.0	106.1	106.7	106.5	106.6	105.5	104.7	99.4	98.3
	Overall RSD, %	8.9	7.1	4.1	2.9	3.3	4.3	5.0	1.7	1.4
Cu[a]	Expected value, mg/L	0.0025	0.005	0.0075	0.0125	0.015	0.050	0.10	0.20	0.30
	Overall recovery, %	101.6	101.9	101.1	102.9	101.9	101.6	100.4	99.3	96.3
	Overall RSD, %	6.8	6.1	3.2	2.8	2.8	1.9	1.8	3.0	3.1
Zn[a]	Expected value, mg/L	0.010	0.020	0.030	0.050	0.060	0.20	0.40	0.60	1.2
	Overall recovery, %	98.6	100.6	99.5	101.1	100.6	99.7	101.2	100.0	98.1
	Overall RSD, %	7.6	6.6	1.9	2.1	2.9	2.0	1.5	2.0	2.0
Mo[a]	Expected value, µg/L	0.40	0.80	1.2	2.0	2.4	8.0	16.0	24.0	48.0
	Overall recovery, %	97.7	101.2	103.7	98.3	100.7	100.7	100.6	99.7	98.8
	Overall RSD, %	7.5	2.9	4.0	3.5	0.8	1.3	1.2	2.2	2.2
Se[b]	Expected value, µg/L	0.20	0.40	0.60	1.0	1.2	4.0	8.0	12.0	24.0
	Overall recovery, %	100.0	97.3	98.2	96.6	99.4	100.0	99.0	99.4	99.9
	Overall RSD, %	3.4	2.8	2.0	3.3	1.4	1.2	0.5	2.2	1.2

[a] He gas mode; Ge ISTD.

[b] H_2 gas mode; Te ISTD.

unfit for validation work. Most elements demonstrated RSD_{iR} <5% for all matrixes, which is quite remarkable considering the opportunities for variability in the study design. There is no SMPR for intermediate precision, but these data suggest that the method would perform well in a collaborative study, and this proved to be the case (*see* below).

The cases for Fe, Cu, and Mn are discussed under LOQ. There were no SPIFAN matrixes that really challenged the method anywhere near the required lower analytical range for these elements, and no low-level spikes were performed.

Precision data from SRM 1849a are shown in Table 5. The SRM was analyzed nine times, on different days and yielded RSD_{iR} of <3% for all elements.

Recovery/Accuracy

The SMPR designates a recovery of 90–110% over the range of the assay, and 80–115% for low levels of Mn. Table 6 shows the recovery of each element in each SPIFAN matrix measured in triplicate over each of 3 days. The spikes were added at approximately 100% of the nominal element concentration, and the triplicate means on each day were averaged to one result

Table 3. Calibration standards and PLOQ

	Na, mg/L	Mg, mg/L	P, mg/L	K, mg/L	Ca, mg/L	Mn, mg/L	Fe, mg/L	Cu, mg/L	Zn, mg/L	Cr, µg/L	Se, µg/L	Mo, µg/L
Cal Blk	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Cal Std 1	0.500	0.200	0.500	1.00	1.00	0.00500	0.0500	0.00500	0.0200	0.800	0.400	0.800
Cal Std 2	2.50	1.00	2.50	5.00	5.00	0.0250	0.250	0.0250	0.100	4.00	2.00	4.00
Cal Std 3	10.0	4.00	10.0	20.0	20.0	0.100	1.00	0.100	0.400	16.0	8.00	16.0
Cal Std 4	20.0	8.00	20.0	40.0	40.0	0.200	2.00	0.200	0.800	32.0	16.0	32.0
PLOQ, mg/L[a]	0.25	0.10	0.25	0.50	0.50	0.0025	0.025	0.0025	0.010	0.4	0.2	0.4
PLOQ, mg/100 g[b]	1.3	0.50	1.3	2.5	2.5	**0.013**[c]	**0.13**[c]	**0.013**[c]	0.050	20	10	20
SMPRs, mg/100 g[d]	10	3	15	10	20	0.001	0.01	0.001	0.1	20	10	20

[a] Units are µg/L for Cr, Mo, and Se.

[b] mg of Na, Mg, P, K, Ca, Mn, Fe, Cu, or Zn/100 g of reconstituted final product, or µg of Cr, Mo, or Se/kg of reconstituted final product for a typical dilution factor of 50:1.0 g RTF product or reconstituted powder/50 mL final volume.

[c] Note that the PLOQs for Mn, Fe, and Cu (in boldface) do not meet SMPR requirements.

[d] mg of Na, Mg, P, K, Ca, Mn, Fe, Cu, or Zn/100 g of reconstituted final product, or µg of Cr, Mo, or Se/kg of reconstituted final product.

Table 4. Summary of results for RSD_{ip} in 11 SPIFAN matrixes tested in duplicate over 8 days (results were collected in terms of "per kg" as-is for powders in this case, rather than the default SPIFAN units of mg/100 g or µg/100 g reconstituted product)

Matrix/SPIFAN No.	Parameter	Na	Mg	P	K	Ca	Cr[a]	Mn	Fe	Cu	Zn	Se[a]	Mo[a]
A-RTF, high fat 00406RF00[b]	Mean, mg/kg	1390	398	1010	2360	888	141	4.80	21.5	2.37	27.9	133	193
	RSD, %	2.0	1.8	2.9	1.7	9.4	1.8	4.8	3.6	3.0	1.4	3.6	2.6
A-RTF, high protein 00414RF00	Mean, mg/kg	1020	330	951	1560	983	130	4.22	18.7	1.81	22.0	92.6	154
	RSD, %	1.9	3.4	3.4	3.9	2.8	2.9	2.1	3.4	3.0	2.4	3.0	3.2
AP, milk protein 11750017V3[c]	Mean, mg/kg	2270	430	2140	5810	2820	142	5.30	43.8	11.5	52.4	219	294
	RSD, %	2.2	3.3	2.4	1.7	2.3	5.0	2.6	4.0	3.3	2.6	2.8	4.9
AP, low fat 00394RF00	Mean, mg/kg	2070	1130	2710	5060	2740	428	14.0	59.8	6.34	62.1	268	565
	RSD, %	2.8	2.9	1.8	2.1	2.7	1.3	2.7	3.5	3.3	2.6	3.2	3.0
PP 00412RF00[d]	Mean, mg/kg	1460	741	3490	5500	3790	271.9	7.62	57.7	5.15	37.6	213	261
	RSD, %	2.6	2.5	2.0	2.5	1.8	1.3	1.4	3.2	3.7	1.7	2.9	5.1
IP, elemental 00403RF00[e]	Mean, mg/kg	2410	489	4560	7970	6310	215	4.67	105	7.20	64.6	209	160
	RSD, %	3.0	3.3	2.4	3.1	2.2	6.3	1.3	1.9	1.7	2.1	2.8	7.5
IP, milk D04HTCVV	Mean, mg/kg	1870	565	2800	6750	4690	44.3	1.05	116	5.43	65.9	230	159
	RSD, %	3.2	2.9	1.7	3.3	2.5	7.7	2.0	2.0	4.9	1.5	3.8	8.2
IP, milk, partially hydrolyzed 1172572116	Mean, mg/kg	1560	377	2310	6450	4190	21.4	1.05	83.7	4.90	43.4	239	184
	RSD, %	1.8	2.6	4.4	1.1	2.2	20.2	2.6	3.1	3.9	2.6	2.7	4.0
IP, soy, partially hydrolyzed 117257651Z	Mean, mg/kg	2470	604	3950	7400	6480	53.6	2.30	111	4.88	49.2	242	297
	RSD, %	2.9	2.9	3.6	2.2	2.2	9.2	2.6	2.5	3.9	1.8	4.1	3.6
I-RTF, soy E29JVLV[f]	Mean, mg/kg	2301.3	726	4250	7540	6470	72.5	3.31	114	5.40	73.9	223	328
	RSD, %	2.4	2.8	5.0	2.6	3.5	5.2	2.3	2.4	4.1	2.3	3.3	4.5
I-RTF, milk control	Mean, mg/kg	179	59.4	298	1000	598	7.61	0.153	14.3	0.579	7.17	29.6	16.9
	RSD, %	2.2	3.1	4.3	3.0	4.5	52.7	2.9	4.3	3.5	2.1	2.9	10.0

[a] Concentrations in µg/kg.

[b] A-RTF = Adult ready-to-feed formula.

[c] AP = Adult powder formula.

[d] PP = Pediatric powder formula.

[e] IP = Infant powder formula.

[f] I-RTF = Infant ready-to-feed formula.

Table 5. Precision and accuracy with SRM 1849a

		SRM 1849a		Candidate method				
Analyte	Units	Certified mean	Certified range	*n*	Mean	Bias	Accuracy, %	RSD_{ir}, %
Na	mg/100 g	426.5	418.2–434.8	9	429	2.5	100.6	0.98
Mg	mg/100 g	164.8	161.2–168.4	9	163	–2.1	98.7	2.2
P	mg/100 g	399.0	385.0–413.0	9	430	30.6	107.7	1.4
K	mg/100 g	922.0	911.0–933.0	9	929	6.6	100.7	1.9
Ca	mg/100 g	525.3	520.2–530.4	9	534	8.7	101.7	1.4
Cr	µg/100 g	107.2	104.0–110.4	9	105	–2.5	97.7	2.3
Mn	mg/100 g	4.959	4.8–5.056	9	4.85	–0.1	98.6	2.1
Fe	mg/100 g	17.56	17.27–17.85	9	17.4	–0.2	99.1	1.2
Cu	mg/100 g	1.978	1.952–2.004	9	1.93	–0.1	97.6	2.7
Zn	mg/100 g	15.1	14.54–15.66	9	15.4	0.3	102.0	2.0
Se	µg/100 g	81.2	78.3–84.1	9	81.7	0.5	100.6	1.0
Mo	µg/100 g	170.7	166.7–174.7	9	164	–6.5	96.2	1.2

Table 6. Recovery in SPIFAN matrixes

Matrix/ SPIFAN No.	Parameter	Na	Mg	P	K	Ca	Cr	Mn	Fe	Cu	Zn	Se	Mo
A-RTF, high fat 00406RF00[a]	Recovery, %	100.2	99.2	109.0	105.1	104.1	98.0	99.7	99.3	105.7	108.0	105.2	93.3
	RSD, %	4.4	9.0	2.8	8.2	14.1	2.8	8.0	9.1	6.3	11.4	7.0	3.1
A-RTF, high protein 00414RF00	Recovery, %	105.2	92.8	100.2	92.4	98.6	98.0	94.3	97.3	91.3	94.1	103.6	92.0
	RSD, %	5.9	9.9	5.5	11.1	8.4	2.5	4.4	8.1	4.1	2.8	4.9	2.7
AP, milk protein 11750017V3[b]	Recovery, %	105.4	104.2	100.0	102.6	100.7	103.7	99.5	98.3	104.6	97.4	102.8	97.4
	RSD, %	2.7	4.0	2.9	1.3	4.2	1.6	5.6	3.7	1.4	4.1	4.1	4.6
AP, low fat 00394RF00	Recovery, %	102.8	102.0	98.1	107.9	99.6	100.5	100.5	98.8	99.3	97.3	99.3	95.4
	RSD, %	5.5	8.3	6.8	5.3	8.0	4.8	4.8	7.4	4.0	5.3	3.3	8.1
PP 00412RF00[c]	Recovery, %	105.6	104.0	106.2	104.6	103.2	101.5	95.3	101.1	95.3	98.0	103.6	96.2
	RSD, %	6.8	9.8	3.2	10.6	7.5	2.5	0.7	2.2	0.7	1.1	4.8	2.1
IP, elemental 00403RF00[d]	Recovery, %	105.7	100.2	107.3	99.2	101.1	101.5	99.6	99.8	99.4	97.0	105.5	96.9
	RSD, %	10.8	4.5	1.3	10.4	12.5	8.8	5.6	5.2	1.4	3.8	4.6	2.7
IP, milk D04HTCVV	Recovery, %	102.2	100.8	102.2	104.4	98.6	108.8	99.6	98.4	89.8	98.9	105.6	92.5
	RSD, %	4.5	9.0	6.8	16.0	3.5	10.3	5.5	4.6	2.1	3.2	4.7	2.5
IP, milk, partially hydrolyzed 1172572116	Recovery, %	101.3	103.5	101.5	100.5	98.4	90.1	98.9	97.1	102.4	96.8	99.5	95.2
	RSD, %	2.3	5.2	6.8	7.1	8.6	1.1	7.0	6.3	8.6	5.8	2.3	5.7
IP, soy, partially hydrolyzed 117257651Z	Recovery, %	99.8	103.3	100.1	98.0	100.2	91.0	101.2	96.8	97.8	95.2	101.2	107.9
	RSD, %	3.6	11.3	2.7	6.6	9.7	3.0	0.6	6.5	4.8	4.5	2.3	1.0
I-RTF, soy E29JVLV[e]	Recovery, %	105.6	103.3	107.6	110.2	102.5	107.8	94.9	96.9	93.8	95.4	102.9	93.1
	RSD, %	7.6	8.8	5.3	11.8	11.8	1.5	3.5	4.6	1.0	2.7	6.0	1.2
I-RTF, milk control	Recovery, %	103.2	93.0	99.9	108.4	90.2	103.3	93.5	92.8	91.7	93.0	100.7	92.3
	RSD, %	5.3	2.7	2.9	13.5	4.0	6.1	2.2	3.8	2.9	1.5	4.9	5.4

[a] A-RTF = Adult ready-to-feed formula.

[b] AP = Adult powder formula.

[c] PP = Pediatric powder formula.

[d] IP = Infant powder formula.

[e] I-RTF = Infant ready-to-feed formula.

Table 7. Straight % RSDs of six or seven laboratory results from the MLT (no outliers removed; includes SLV means)

	Na	Mg	P	K	Ca	Mn	Fe	Cu	Zn
No. of laboratories	6	6	6	6	6	7	7	7	7
Adult milk powder	6.6	6.9	7.6	3.9	5.1	3.6	4.7	3.0	5.8
Infant powder hydrolyzed milk	6.8	6.8	8.1[a]	3.3	5.2	3.2	3.4	2.1	1.9
Adult powder low fat	6.4	6.5	8.3[a]	3.6	5.7	3.0	4.8	2.4	5.3
Child powder	6.6	7.3	7.3	4.2	5.1	3.4	4.6	2.6	2.4
Infant elemental powder	6.2	6.5	4.4	4.0	5.5	3.5	4.8	2.3	5.9
Average of five matrixes	6.5	6.8	7.1	3.8	5.3	3.3	4.5	2.5	4.3
Adult RTF high protein	7.6	8.1	14.0[a]	4.8	33.8[a]	25.5[a]	11.9[a]	2.8	14.2[a]
Adult RTF high fat	8.5[a]	7.9	10.9[a]	5.1	48.1[a]	26.1[a]	8.9	3.4	9.7
SRM 1849a	2.4	3.0	1.9	1.7	1.5	4.0	3.8	2.0	2.0
SMPR required RSD_R	8	10	8	8	8	10	10	10	10

[a] Would fail the SMPR criterion for reproducibility.

before taking the ($n = 3$) statistics shown in Table 6. All elements in all matrixes had average spike recoveries in the 90–110% range (with rounding), and so the SMPR was met for recovery. Again, the method was not challenged for the low levels of Cu, Fe, or Mn in this regard.

Table 5 shows the accuracy of average values from nine determinations for each element in SRM 1849a. Accuracies ranged from 96.2% (Mo) to 107.7% (P), in agreement with the spike recovery results. Only Ca, P, Cu, and Mo produced results outside of the certified range, but the results were consistent with the MLT results from other laboratories and with ICP-AES results (*see* below).

It should be noted that during these studies Ni was shown to be an acceptable alternative to Ge as an ISTD (data not shown), but due to the significant concentration of Ni in cocoa products, Ge was chosen as the ISTD for the method (except for the use of Te for Se determinations in the H_2 gas mode).

MLT Study and ICP-AES Comparative Data

The same laboratories that participated in the MLT study of Cr, Mo, and Se (OMA **2011.19**; 6) were asked to provide data for the other nine elements of this present study. Five laboratories provided results for Na, Mg, P, K, and Ca, while six laboratories provided results for Fe, Zn, Cu, and Mn. These laboratories provided two results/matrix because they were provided blind duplicates of each material. The data from the SLV described above provided another point and were averaged in at equal weighting with the other laboratories' data, so that data were collected from 6 to 7 different laboratories in total. Table 7 shows the straight RSDs of the mean results from either 11 or 13 results for each matrix (five laboratories × 2 + SLV, or six laboratories × 2 + SLV). Given the unequal weighting of the source data, the borderline number of laboratories participating, and the fact that no outliers were removed (other than those from failing system suitability), these RSDs are not exactly the reproducibility parameter (RSD_R) but should be a very good estimation of it. The RSDs in Table 7 were very consistent except for the Adult RTF products, which had many disparate results. It is widely believed that these two RTFs were too far past the end of shelf life and were no longer viable to test. With removal of these two products, the RSDs in Table 7 all pass the required reproducibility of the SMPR shown at the bottom of the table with the exception of P, for which two product matrixes were just above the required 8.0% RSD. It can be hypothesized that the RSDs for the low mass, high concentration elements are a little higher than for the trace elements at higher masses (on the right side of Table 7) because of slight differences in how these instruments handled collision/reaction interference removal and how well they performed P/A crossover calibrations. There were four different models of ICP/MS instruments contributing to the data in Table 7: an Agilent

Table 8. Percentage difference of six or seven MLT laboratory mean relative to Abbott 6-day SLV using microwave digestion-ICP-AES

Product type	Na	Mg	P	K	Ca	Mn	Fe	Cu	Zn
SRM 1849a	–0.4	–1.4	2.8	0.3	–1.6	–0.8	1.1	0.1	2.9
Adult milk protein powder	5.5	2.8	3.8	2.7	4.0	4.5	7.2	3.9	8.0
Infant powder hydrolyzed milk	4.4	2.6	2.0	0.7	1.8	6.6	5.8	3.5	4.7
Adult powder low fat	2.3	0.4	1.5	–0.5	1.4	0.5	3.8	0.5	3.8
Child powder	4.7	0.5	5.2	0.8	2.6	3.1	7.0	2.5	5.7
Infant elemental powder	6.0	4.2	6.0	3.0	4.7	4.5	6.5	5.6	5.9
Adult RTF high protein	5.2	2.4	–0.5	1.1	–7.0	–13.8	3.6	–1.7	–2.6
Adult RTF high fat	4.6	1.3	–11.5	1.2	–34.7	–24.5	13.0	0.5	0.4

7500cx from the SLV, a few Agilent 7700x, a PerkinElmer ELAN DRC-e, and a Thermo X Series 2. On the other hand, it is of interest to note the excellent RSDs for Mn and Cu across these laboratories—likely due to the excellent sensitivity of the ICP/MS for these elements and the effectiveness of the CRCs in removing background interferences at somewhat higher mass.

The accuracy of the present method can be further attested to by comparison to an independent method, the commonly used ICP-AES, also with microwave digestion. A full SLV was performed on the SPIFAN matrix set in the authors' laboratory using the same microwave oven (CEM MARS 5 with MARSXpress™ vessels) and two PerkinElmer Optima ICP instruments. The mean 6-day ICP-AES results were compared to the mean values from the ICP/MS MLT (similar to those means in Table 4). The results are shown in Table 8. Again, we must disregard the numbers for the Adult RTFs because the ICP-AES data were acquired several months ahead of the MLT study, and these products had probably physically deteriorated. The remaining powder products show remarkable agreement between the two spectroscopies. In general, MS data are higher than those produced by emission, but seldom is there more than 6% difference.

Conclusions

The method, as is, meets all SMPRs except for the LOQ of Fe, Mn, and Cu. There was also substantial evidence presented to support the accuracy and reproducibility of this method through comparison to an independent method and through analyses completed at independent laboratories with different models of ICP/MS instruments. The data from the SLV and MLT studies were consistent with each other. Additional linearity work, spiking at low-levels, increasing sample size, and/or additional low level standards would be needed to prove accuracy at the lowest levels for Fe, Cu, and Mn.

Acknowledgments

We thank Andre Szabo for participating as the second analyst in this SLV. Also, we thank the participants of the OMA **2011.19** collaborative study that contributed nine-element data for this report: Fan Xiang, Sudhakar Yadlapalli, Isabelle Malaviole, Ashutosh Mittal, Michael Gray, Diana Mould, and Michael Farrow.

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AOAC Official Method 2015.07

Chloride in Infant Formula and Adult/Pediatric Nutritional Formula

Potentiometric Titration Method

First Action 2015

(Applicable for determination of chloride in all forms of infant, adult, and/or pediatric formula including powders, ready to feed liquids, and liquid concentrates.)

Caution: Refer to Material Safety Data Sheets prior to use of chemicals. Use appropriate personal protective equipment when performing testing.

A. Principle

Samples are treated with potassium ferrocyanide and zinc acetate to precipitate protein, acidified with nitric acid, and titrated using silver nitrate as titrant. The endpoint of titration is determined by the potentiometric method on a silver titrode with silver sulfide coated electrode. Chloride content is calculated in the sample via the titrant volume at the endpoint.

B. Apparatus

(a) *Analytical Balances*.-Accurate to 0.1 mg and 0.01 mg.

(b) *Centrifuge*.-Tabletop with rotor to fit 50 mL conical tubes.

(c) *Centrifuge tubes*.-50 mL, conical, polypropylene.

(d) *Pipette*.-10 mL, grade A.

(e) *Volumetric flasks*.-50, 100, 500, and 1000 mL glass.

(f) *Graduated cylinders*.-25 mL, 100 mL, and 500 mL, glass.

(g) *Beaker*.-120 mL, Metrohm sample beaker (6.1459.300) or equivalent.

(h) *Silver titrode*.-Metrohm Ag titrode (6.0430.100) or equivalent.

(i) *Automatic potentiometric titration system*.-Metrohm 862 Compact Titrosampler

(2.862.0010) equipped with 800 Dosino (2.800.0010), 10 mL dosing unit (6.3032.210) and automatic propeller blending device or equivalent.

C. Reagents

(a) *Water*.-Reagent Grade.

(b) *Silver nitrate solution*.-0.1 M standard titrant with certified concentration to 4 significant figures. (c) *Potassium ferrocyanide trihydrate*.-Analytical reagent.

(d) *Zinc acetate dehydrate*.- Analytical reagent.

(e) *Nitric acid*.- Analytical reagent.

(f) *Ethanol*.- Analytical reagent.

(g) *Sodium chloride*.- Reference reagent.

D. Preparation of Solutions

(a) *Precipitating agent I*.- Dissolve 106 g potassium ferrocyanide trihydrate and bring to 1000 mL using water.

(b) *Precipitating agent II*.-Dissolve 220 g zinc acetate dehydrate and bring to 1000 mL using water.

(c) *Nitric acid solution*.-Add 100 mL nitric acid to 300 mL water and mix well.

(d) *Washing solution*.-Add 75 mL ethanol to 25 mL water and mix well.

(e) *Sodium chloride standard solution (NaCl SS)*.-Weigh 250 mg (accurate to 0.01 mg) NaCl and dissolve in water to total solution weight of 25 g (accurate to 0.1 mg). Mix well. Prepare fresh before the titer check.

E. Sample Preparation

(a) Weigh 5 g (accurate to 0.1 mg) powder sample (2 g for skim milk powder) or 20 g (accurate to 0.1 mg) liquid sample in 50 mL centrifuge tube. Add 25 mL 40°C water for powder sample and dissolve thoroughly.

(b) Transfer 2.5 mL precipitating agent I and 2.5 mL precipitating agent II into the tube, bring to 50 mL with water and mix well.

(c) Centrifuge at 12500 x *g* for 5 min at 4°C (6 min for skim milk powder) and equilibrate to room temperature.

(d) Accurately transfer 10 mL supernatant (20 mL for desalted whey powder D90) into a 120 mL sample beaker, add 5 mL nitric acid solution and 50 mL water before titration.

F. System Suitability

(a) Weigh 1000 - 1500 mg (accurate to 0.1 mg) NaCl SS into a 120 mL sample beaker.

(b) Add 5 mL nitric acid solution and 50 mL water.

(c) Put the washing solution in the washing position of the auto sampler, and replace with fresh washing solution after every 10 or 11 single titration tests.

(d) The titration conditions for system suitability analysis are presented in Table **2015.07**.

(e) Titrate using the titrator.

(f) Calculate concentration of the silver nitrate solution according to section ***H***(a). The difference between the calculated concentration and the certified value should be within 0.5% . If outside the acceptance value, check the experimental procedures and titration system. If issue is not resolved, use fresh silver nitrate. If fresh silver nitrate cannot be able to gain a acceptable range, replace the electrolyte of the electrode and check the condition of dosing unit.

G. Analysis

(a) Titrate the prepared sample solution on the titrator. Put the washing solution in special washing position of the auto sampler, and use change fresh washing solution every 10 or 11 single titration tests. The sample titration conditions are the same as the system suitability analysis in Table **2015.07**.

H. Calculations

(a) Calculate silver nitrate concentration (SNC) in mol/L for system suitability verification and report to 4 decimal places:

$$\text{SNC (mol/L)} = \frac{m_1}{5.844} \times \frac{m_2}{m_3} \times \frac{1}{V_1} \times \frac{1}{10}$$

where m_1 = weight of sodium chloride standard solution (mg); m_2 = weight of sodium chloride used to prepare the standard solution (mg); m_3 = total weight of prepared sodium chloride standard solution (mg); V_1 = $AgNO_3$ consumption volume up to titration endpoint (mL); 5.844 = sodium chloride weight in µg corresponding to 1 mL of 0.1 mol/L $AgNO_3$; and 10 = mass conversion from titer to the concentration of titrant.

(b) Calculate chloride content in sample (CL) and report to 3 significant digits:

$$\text{CL (mg/100g)} = \frac{35.5 \times c \times V_2 \times f \times 100}{m_4}$$

where m_4 = sample weight (g); c = certified concentration of silver nitrate titrant (mol/L); V_2 = $AgNO_3$ consumption volume up to titration endpoint (mL); f = dilution factor; 35.5 = chloride weight in µg corresponding to 1 mL of 1 mol/L $AgNO_3$; and 100 = mass conversion to mg/100 g.

References: *J. AOAC Int*. (future issue)

AOAC SMPR 2014.015

J. AOAC Int. **98**, 1079(2015)

Posted: October 1, 2015

Table 2015.07. Titration Conditions for System Suitability Test (DET U mode) and Sample Analysis

Module / Parameter	Condition
Start conditions / Pause	15 s
Titration parameters / Measure point density	4
Titration parameters / Minimum increment	10.0 µL
Titration parameters / Dosing Rate	Max. mL/min
Titration parameters / Signal drift	50 mV/min
Titration parameters / Equilibrium time	26 s
Titration parameters / Measure input	1
Titration parameters / Stirrer rate	10
Stop conditions / Stop volume	10 mL
Stop conditions / Stop measure value	120 mV
Stop conditions / Stop EP	1
Stop conditions / Volume after EP	1 mL
Evaluation / EP recognition	greatest
Reports / PC/LIMS	on
Automation / Dripping time	3 s
Automation / Rinsing time	15 s
Automation / Stirring rate	10

INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Chloride in Infant Formula and Adult/Pediatric Nutritional Formula by Potentiometric Titration: Single-Laboratory Validation, First Action 2015.07

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A potentiometric method for determination of chloride was validated against AOAC *Standard Method Performance Requirement* (SMPR®) 2014.015. Ten AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) matrixes, including National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1849a, were tested in duplicate on 6 independent days. The repeatability (RSD_r) ranged from 0.43 to 1.34%, and the intermediate reproducibility (RSD_{iR}) ranged from 0.80 to 3.04%. All results for NIST SRM 1849a were within the range of the certified concentration (701±17 mg/100 g). Recovery was demonstrated with two overspike levels, 50 and 100%, in the 10 SPIFAN matrixes. Samples were tested in duplicate on 3 different days, and all results were within the SMPR requirement of 95 to 105%. The LOQs of the method for powdered products and ready-to-feed or reconstituted products were 20 mg/100 g and 2.2 mg/100 mL, respectively. A wide analytical range from the LOQ to 99.5% chlorine content can be reached with an appropriate dilution factor, but in practice, the upper analytical value observed in routine matrix testing was approximately 1080 mg/100 g in skim milk powder. This is a rapid, simple, and reliable chlorine-testing method applicable to infant formula, adult nutritionals, and ingredients used in these dairy-based products, such as skim milk powder, desalted whey powder, whey protein powder, and whole milk powder.

In response to a need for a reference method for dispute resolution, the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) developed *Standard Method Performance Requirement* (SMPR®) 2014.015, "*Standard Method Performance Requirements* for Determination of Chloride in Infant and Adult/Pediatric Nutritional Formula" (1). National Food Safety Standard GB 5413.24-2010, "Determination of chlorine in foods for infants and young children, milk and milk products," is a Chinese nationally enforced testing method published by the Ministry of Health in 2010 as Notice 7. The notice includes 66 national standards in the area of dairy products and forms the regime of Chinese dairy product safety national standards. There are two methods within GB 5413.24-2010: one is a potentiometric titration method, and the other is a traditional titration method using a color indicator to determine the end of titration. The potentiometric titration method in GB 5413.24-2010 has a limitation in the end point determination or in precise titrant volume recording due to being an older titration technique using older instrumental analysis. A new potentiometric titration method was therefore developed at the Comprehensive Test Center of Chinese Academy of Inspection and Quarantine (CAIQTEST), which applied a modern, sophisticated, automatic titration system for enhancing the precision, accuracy, and efficiency of testing. It is a high-throughput, practical method that can be used in routine testing.

CAIQTEST is a national institute under the leadership of Chinese Academy of Inspection and Quarantine (CAIQ) and operating as a third-party inspection agency in accordance with ISO/IEC 17025. CAIQ is a national public institute for researching and developing science and technology to be applied in inspection and quarantine. The mission of CAIQ is mainly to conduct research on the applied science of inspection and quarantine, as well as basic, high-tech, and soft science, with the focus on solving general and comprehensive problems and emergent and pivotal issues related to the administration of inspection and quarantine. CAIQ provides technical support to the policy making related to inspection and quarantine for China's central government, and provides technical assistance to the law enforcement duties of the General Administration of Quality Supervision, Inspection, and Quarantine.

Single-Laboratory Validation Study

The validation study compared the results of the method to the criteria of AOAC SMPR 2014.015. The requirements are presented in Table 1. Validation experiments included determination of system suitability, precision, accuracy, LOQ, and analytical range of the method.

Received June 01, 2015. Accepted by AK July 08, 2015.
This method was approved by the Expert Review Panel for Infant Formula and Adult Nutritionals as First Action.
The Expert Review Panel for Infant Formula and Adult Nutritionals invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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DOI: 10.5740/jaoacint.15-0143

Table 1. Criteria of SMPR 2014.015

Parameter	Minimum acceptable criteria
Analytical range	5–500[a,b]
LOQ	≤5[a,b]
Accuracy	95–105%
Repeatability (RSD_r)	≤2%
Reproducibility (RSD_R)	≤4%

[a] Concentrations apply to (*1*) "ready-to-feed" liquids "as-is"; (*2*) reconstituted powders (25 g into 200 g water); and (*3*) liquid concentrate diluted 1:1 by weight.

[b] Milligrams per 100 g in reconstituted final product.

AOAC Official Method 2015.07 Chloride in Infant Formula and Adult/Pediatric Nutritional Formula

Potentiometric Titration Method

First Action 2015

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A. Principle

Samples are treated with potassium ferrocyanide and zinc acetate to precipitate protein, acidified with nitric acid, and titrated using silver nitrate as titrant. The end point of titration is determined by the potentiometric method on a silver titrode with a silver sulfide-coated electrode. Chloride content is calculated in the sample via the titrant volume at the end point.

B. Apparatus

(a) *Analytical balances*.—Accurate to 0.1 and 0.01 mg.

(b) *Centrifuge*.—Tabletop with rotor to fit 50 mL conical tubes.

(c) *Centrifuge tubes*.—50 mL, conical, polypropylene.

(d) *Pipet*.—10 mL, grade A.

(e) *Volumetric flasks*.—50, 100, 500, and 1000 mL, glass.

(f) *Graduated cylinders*.—25, 100, and 500 mL, glass.

(g) *Beaker*.—120 mL sample beaker (Metrohm, Herisau, Switzerland 6.1459.300 or equivalent) (Metrohm).

(h) *Silver titrode*.—Ag titrode (Metrohm 6.0430.100 or equivalent).

(i) *Automatic potentiometric titration system*.—Metrohm 862 Compact Titrosampler (2.862.0010) equipped with 800 Dosino (2.800.0010), 10 mL dosing unit (6.3032.210), and automatic propeller blending device or equivalent.

C. Reagents

(a) *Water*.—Reagent grade.

(b) *Silver nitrate solution*.—0.1 M standard titrant with certified concentration to 4 significant figures.

(c) *Potassium ferrocyanide trihydrate*.—Analytical reagent.

(d) *Zinc acetate dehydrate*.—Analytical reagent.

(e) *Nitric acid*.—Analytical reagent.

(f) *Ethanol*.—Analytical reagent.

(g) *Sodium chloride*.—Reference reagent.

D. Preparation of Solutions

(a) *Precipitating agent I*.—Dissolve 106 g potassium ferrocyanide trihydrate and dilute to 1000 mL using water.

(b) *Precipitating agent II*.—Dissolve 220 g zinc acetate dihydrate and dilute to 1000 mL using water.

(c) *Nitric acid solution*.—Add 100 mL nitric acid to 300 mL water and mix well.

(d) *Washing solution*.—Add 75 mL ethanol to 25 mL water and mix well.

(e) *Sodium chloride standard solution (NaCl SS)*.—Weigh 250 mg (accurate to 0.01 mg) NaCl and dissolve in water to total solution weight of 25 g (accurate to 0.1 mg). Mix well. Prepare fresh before the titer check.

E. Sample Preparation

Weigh 5 g (accurate to 0.1 mg) powder sample (2 g for skim milk powder) or 20 g (accurate to 0.1 mg) liquid sample in 50 mL centrifuge tube. Add 25 mL 40°C water for powder sample and dissolve thoroughly. Transfer 2.5 mL precipitating agent I and 2.5 mL precipitating agent II into the tube, dilute to 50 mL with water, and mix well. Centrifuge at 12 500 × *g* for 5 min at 4°C (6 min for skim milk powder) and equilibrate to room temperature. Accurately transfer 10 mL supernatant (20 mL for desalted whey powder D90) into 120 mL sample beaker and add 5 mL nitric acid solution and 50 mL water before titration.

F. System Suitability

Weigh 1000 to 1500 mg (accurate to 0.1 mg) NaCl SS into 120 mL sample beaker. Add 5 mL nitric acid solution and 50 mL water. Place the washing solution in the washing position of the autosampler and replace with fresh washing solution after every 10 or 11 single titration tests. The titration conditions for system suitability analysis are presented in Table **2015.07**. Titrate using the titrator. Calculate concentration of the silver nitrate solution according to **H**. The difference between the calculated concentration and the certified value should be within 0.5%. If outside the acceptance value, check the experimental procedures and titration system. If the issue is not resolved, use fresh silver nitrate. If fresh silver nitrate is not able to gain an acceptable range, replace the electrolyte of the electrode and check the condition of the dosing unit.

G. Analysis

Titrate the prepared sample solution on the titrator. Place the washing solution in special washing position of the auto sampler, and use fresh washing solution every 10 or 11 single titration tests. The sample titration conditions are the same as the system suitability analysis in Table **2015.07**.

H. Calculations

Calculate silver nitrate concentration (SNC) in moles per liter for system suitability verification and report to 4 decimal places:

$$\mathrm{SNC}\left(\mathrm{mol/L}\right) = \frac{m_1}{5.844} \times \frac{m_2}{m_3} \times \frac{1}{V_1} \times \frac{1}{10}$$

where m_1 = weight in milligrams of NaCl SS, m_2 = weight in milligrams of sodium chloride used to prepare the standard solution, m_3 = total weight in milligrams of prepared NaCl SS, V_1 = silver nitrate consumption volume in milliliters up to titration end point, 5.844 = sodium chloride weight in micrograms corresponding to 1 mL of 0.1 mol/L silver nitrate, and 10 = mass conversion from titer to the concentration of titrant.

Calculate chloride content in sample (CL) and report to 3 significant digits:

$$\mathrm{CL}\left(\mathrm{mg/100g}\right) = \frac{35.5 \times c \times V_2 \times f \times 100}{m_4}$$

where m_4 = sample weight in grams, c = certified concentration in moles per liter of silver nitrate titrant, V_2= silver nitrate consumption volume in milliliters up to titration end point, f = dilution factor, 35.5 = chloride weight in micrograms corresponding to 1 mL of 1 mol/L silver nitrate, and 100 = mass conversion to milligrams per 100 g.

See refs. 2–4 for more detail.

Table 2015.07. Titration conditions for system suitability test (dynamic equivalence-point titration U mode) and sample analysis

Module and parameter	Condition
Start conditions	
Pause	15 s
Titration parameters	
Measure point density	4
Minimum increment	10.0 µL
Dosing rate	Maximum mL/min
Signal drift	50 mV/min
Equilibrium time	26 s
Measure input	1
Stirrer rate	10
Stop conditions	
Stop volume	10 mL
Stop measure value	120 mV
Stop equivalence point	1
Volume after equivalence point	1 mL
Evaluation	
Equivalence-point recognition	Greatest
Reports	
PC/LIMS[a]	On
Automation	
Dripping time	3 s
Rinsing time	15 s
Stirring rate	10

[a] PC/LIMS = Personal computer/laboratory information management system.

Results and Discussion

System Suitability

NaCl SS was prepared as in Table 2 and used to determine the SNC of the silver nitrate solution as described in the method. Titrations were carried out on 3 days in duplicate, and results are shown in Table 3. The calculated concentrations were all within 5% of the certified value of 0.1004 mol/L as required by the method. Thus, system suitability was achieved.

Precision

Two samples each of 10 SPIFAN matrixes from previous multilaboratory studies, including National Institute of Standards and Technology Standard Reference Material (SRM) 1849a, were tested on 6 different days. The results are presented in Table 4. Average within-day repeatability (RSD_r) values for the 10 matrixes varied from 0.43 to 1.34%, meeting the SMPR criterion of ≤2%. Interday intermediate reproducibility varied from 0.80 to 3.04% across the 10 matrixes, in agreement with the reproducibility requirement of ≤4%, suggesting that the method may meet the criterion in a multilaboratory validation.

Table 2. NaCl stock solution preparation

Substance	Amount
NaCl, mg	255.43
NaCl SS	
Total weight, mg	25049.0
Concentration, mg NaCl/g solution	10.20

Table 3. System suitability results for silver nitrate solution with a certified concentration of 0.1004 mol/L

Test round	Duplicate	Mass of NaCl SS, mg	NaCl equivalent, mg	Silver nitrate, mL	Calculated SNC, mol/L
1	1	1007.6	10.27	1.7480	0.1006
	2	1043.2	10.64	1.8133	0.1004
2	1	1024.2	10.44	1.7818	0.1003
	2	1205.6	12.29	2.0930	0.1005
3	1	1013.3	10.33	1.7568	0.1006
	2	1071.5	10.93	1.8628	0.1004

Accuracy/Recovery

Accuracy was demonstrated in the precision study with the use of SRM 1849a (Table 4). All results obtained fell within the certified concentration range of the method. The accuracy values ranged from 99.9 to 102.3%, meeting the requirements of SMPR 2014.015.

Samples of 10 SPIFAN matrixes spiked at two levels (50 and 100% overspikes) were prepared and tested in duplicate on 3 days for recovery. Results are presented in Table 5. Within-day recoveries across the matrixes varied between 100.3 and 103.3%, and among-day recoveries varied between 100.9 and 102.5%, all within the 95 to 105% requirement.

LOQ

The minimum silver nitrate consumption end point volume that can be evaluated by the Metrohm potentiometric titration system is in the range 0.0 to 0.05 mL, depending on the instrument sensitivity conditions and day-to-day variation. A 0.05 mL minimum silver nitrate consumption end point volume is more stable than a 0.02 mL condition and is able to be validated via appropriate dilution of the NaCl SS. This translates to an estimated LOQ of 20 mg/100 g chloride on a powder basis and a 2.2 mg/100 mL chloride result on a ready-to-feed basis (25 g milk powder reconstituted to total 225 g).

Table 4. Precision in SPIFAN matrixes and NIST SRM 1849a

Matrix[a]	Results[b] Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Interday mean	Mean RSD_r, %	RSD_{iR}, %
Adult RTF, high fat[c]									
Mean, mg/100 g	159	156	161	166	169	160	162		
RSD_r, %	1.66	1.82	1.02	1.82	1.26	0.44		1.34	3.04
SRM 1849a (701 ± 17 mg/100 g)									
Mean, mg/100 g	716	706	713	705	705	711	709		
RSD_r, %	0.23	1.22	0.56	0.44	0.70	0.20		0.56	0.80
Pediatric powder									
Mean, mg/100 g	342	342	340	350	347	344	344		
RSD_r, %	0.98	1.24	0.13	0.55	1.22	0.82		0.82	1.23
Adult powder, milk-protein-based									
Mean, mg/100 g	321	312	311	325	318	319	318		
RSD_r, %	1.05	0.89	0.26	1.80	1.11	0.89		1.00	1.77
Infant powder, soy-based									
Mean, mg/100 g	516	521	509	524	517	518	517		
RSD_r, %	0.76	0.23	1.09	0.10	0.14	0.27		0.43	1.01
Infant RTF, milk-based									
Mean, mg/100 g	44.9	45.3	46.5	45.5	45.6	46.3	45.7		
RSD_r, %	0.16	1.58	1.14	1.03	1.24	0.46		0.94	1.45
Adult powder, low-fat									
Mean, mg/100 g	354	339	347	366	355	359	353		
RSD_r, %	0.13	1.69	1.14	0.43	1.80	0.99		1.03	2.66
Adult RTF, high protein									
Mean, mg/100 g	153	157	156	160	160	157	157		
RSD_r, %	0.35	1.47	1.45	1.93	0.44	1.36		1.17	1.78
Infant powder, elemental									
Mean, mg/100 g	360	353	354	373	367	371	363		
RSD_r, %	0.49	1.26	0.60	0.99	0.96	0.38		0.78	2.34
Infant powder, partially hydrolyzed soy-based									
Mean, mg/100 g	419	415	413	427	428	421	421		
RSD_r, %	0.21	1.57	0.24	0.42	0.66	0.67		0.63	1.53

[a] Two samples each of the 10 SPIFAN matrixes were tested.

[b] Concentrations on an "as-is" basis.

[c] RTF = Ready-to-feed.

Table 5. Recovery in SPIFAN matrixes and NIST SRM 1849a

Product[a]	Native concn (RTF or reconstituted, mg/100 g)	Spike recovery			
		Spike level 1 (50%)		Spike level 2 (100%)	
		Avg., %	RSD, %	Avg., %	RSD, %
Two replicates in 3 different days					
Adult RTF, high-fat[b]	162	101.2	0.98	102.5	1.04
SRM 1849a	70.9	101.8	1.32	101.2	0.79
Pediatric powder	38.2	102.2	0.98	102.1	1.28
Adult powder, milk-protein-based	35.3	101.7	1.50	101.2	0.67
Infant powder, soy-based	57.4	101.8	0.94	102.3	1.05
Infant RTF, milk-based	45.7	101.4	0.91	101.8	1.09
Adult powder, low-fat	39.2	100.9	0.76	102.0	1.18
Adult RTF, high protein	157	101.3	0.75	102.5	1.09
Infant powder, elemental	40.3	102.0	0.80	101.6	0.95
Infant powder, partially hydrolyzed soy-based	46.8	101.5	1.21	102.2	1.10
Day 1					
Adult RTF, high-fat	162	101.0	1.40	101.8	1.29
SRM 1849a	70.9	101.8	1.75	101.2	1.40
Pediatric powder	38.2	102.3	0.95	102.8	0.64
Adult powder, milk-protein-based	35.3	101.5	2.16	101.2	0.83
Infant powder, soy-based	57.4	101.5	0.02	101.3	1.33
Infant RTF, milk-based	45.7	100.3	0.79	100.8	0.56
Adult powder, low-fat	39.2	100.7	1.22	101.7	1.17
Adult RTF, high protein	157	101.4	0.03	102.1	2.20
Infant powder, elemental	40.3	101.7	1.29	101.6	1.88
Infant powder, partially hydrolyzed soy-based	46.8	102.6	1.06	101.6	0.06
Day 2					
Adult RTF, high-fat	162	101.5	1.60	103.1	1.22
SRM 1849a	70.9	101.3	2.12	101.2	0.93
Pediatric powder	38.2	102.9	0.18	102.7	1.63
Adult powder, milk-protein-based	35.3	102.1	0.90	100.6	0.60
Infant powder, soy-based	57.4	102.8	1.00	102.7	0.47
Infant RTF, milk-based	45.7	101.8	0.22	102.5	1.38
Adult powder, low-fat	39.2	101.2	0.20	101.1	0.21
Adult RTF, high protein	157	100.8	0.93	103.0	0.40
Infant powder, elemental	40.3	102.2	0.87	101.5	0.85
Infant powder, partially hydrolyzed soy-based	46.8	100.9	1.55	102.0	1.46
Day 3					
Adult RTF, high-fat	162	101.2	0.08	102.6	0.86
SRM 1849a	70.9	102.4	0.23	101.3	0.49
Pediatric powder	38.2	101.5	1.47	100.8	0.49
Adult powder, milk-protein-based	35.3	101.4	2.31	101.6	0.49
Infant powder, soy-based	57.4	101.0	0.52	102.9	0.84
Infant RTF, milk-based	45.7	102.1	0.13	102.1	0.78
Adult powder, low-fat	39.2	100.7	1.04	103.3	0.91
Adult RTF, high protein	157	101.6	1.13	102.3	0.32
Infant powder, elemental	40.3	102.2	0.67	101.6	0.47
Infant powder, partially hydrolyzed soy-based	46.8	101.2	0.85	102.8	1.57

[a] Two samples each of the 10 SPIFAN matrixes were tested.

[b] RTF = Ready-to-feed.

In routine testing, desalted whey powder D90 has been found to have the lowest chloride content. Data from routine testing of this product type, with sample identity blinded, are shown in Table 6. Precision across the three samples meets the reproducibility requirement, validating the LOQ in the range 27.8 to 29.5 mg/100 g. The validated LOQ does not meet the SMPR requirement of ≤5 mg/100 g, but this cannot be tested without appropriately low samples.

Analytical Range

Commercial table salt can be used to test the upper limit of the analytical range of the potentiometric titration method, demonstrating an upper limit of 99.0 to 99.5% (data not shown). In routine testing, however, the matrix with the highest observed chloride content is skim milk powder. Data from routine testing of skim milk powder, with sample identity blinded, are presented in Table 7. The practical analytical range based on real-world samples is 28.5 to 1080 mg/100 g on an "as is" basis (although the practical upper analytical range is likely much higher) and meets the requirement of SMPR 2014.015 of 5 mg/100 g to 500 mg/100 g in ready-to-feed or reconstituted basis.

Table 6. LOQ determination using desalted whey powder

Sample No.	Average result, mg/100 g	Reproducibility (RSD), %
1	27.8	
2	28.2	3.1
3	29.5	

Table 7. Skim milk powder data

Sample No.	Average result, mg/100 g	Reproducibility (RSD), %
1	1070	
2	1080	3.0
3	1020	

Conclusions

The data presented from this single-laboratory validation study demonstrate that the method meets the criteria outlined in SMPR 2014.015 and supports First Action status of the method.

References

(1) *Official Methods of Analysis* (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, Rockville MD, AOAC SMPR 2014.015, www.eoma.aoac.org

(2) GB 5413.24-2010, National Food Safety Standard Determination of Chlorine in Foods for Infants and Young Children, Milk and Milk Products, http://www.moh.gov.cn/zwgkzt/psp/201005/47460.shtml

(3) GB/T 6682-2008, Water for Analytical Laboratory Use—Specification and Test Methods, http://www.sac.gov.cn/SACSearch/search?channelid=97779&templet=gjcxjg_detail.jsp&searchword=STANDARD_CODE=%27GB/T%206682-2008%27&XZ=T

(4) GB/T 601-2002, Chemical Reagent—Preparations of Standard Volumetric Solutions, http://www.sac.gov.cn/SACSearch/search?channelid=97779&templet=gjcxjg_detail.jsp&searchword=STANDARD_CODE=%27GB/T%20601-2002%27&XZ=T.

AOAC Official Method 2015.08
Chloride in Infant Formula and Adult/Pediatric Nutritional Formula

Potentiometry
First Action 2015

[Applicable to the determination of chloride in ready-to-feed (RTF) liquid concentrate and powder products from levels of 1.4 to 1060 mg/100 g reconstituted product or RTF liquids.]

See Tables **2015.08 A–C** for the results of the single-laboratory validation (SLV) study supporting acceptance of the method. The method was evaluated against *Standard Method Performance Requirements* AOAC SMPR® 2014.015.

A. Principle

Reconstitute powder samples by dissolving 25 g powder sample in 200 g warm water (40°C). Add 50 mL of 2% (v/v) nitric acid solution. Stir with a magnetic stirrer until mixed or finely suspended. Ensure pH is <1.5. Titrate potentiometrically against standardized silver nitrate ($AgNO_3$) solution, 0.1 M, using a silver electrode to detect the end point.

B. Apparatus

Common laboratory equipment and, in particular, the following:

(**a**) *Analytical balance.*—Precision 0.1 mg.

(**b**) *Class A volumetric flasks.*—100 and 1000 mL.

(**c**) *pH meter/mV meter with a scale covering ±700 mV, and buret, 20 or 25 mL.*—Mettler-Toledo (Columbus, OH, USA) or equivalent.

(**d**) *Automatic titrator.*—Autosampler, motorized piston buret, with remote-control dispensing and filling (Mettler Toledo T50 Rondo Tower autosampler, Mettler LabX 3.1 software, or equivalent).

(**e**) *Combined ring silver electrode.*—e.g., Mettler Toledo DM 141 or DMi145-SC, or equivalent; alternatively, a silver electrode with reference electrode can be used.

(**f**) *Magnetic stirrer.*—Heidolph MR 3000 or equivalent (Sigma-Aldrich, St. Louis, MO, USA).

(**g**) *Water bath.*—Capable of warming water to 40°C.

(**h**) *Laboratory oven.*—Capable of heating to 120°C.

(**i**) *Pipets (1, 20, 50, and 100 mL).*—Class A glass volumetric or automatic (Eppendorf or equivalent).

(**j**) *Buret.*—10 mL.

C. Chemicals and Reagents

(**a**) *Acetone.*—p.a. (Merck, Darmstadt, Germany, or equivalent).

(**b**) *Water, purified.*—Greater than 18MΩ (EMD Millipore Corp., Billerica, MA, USA, or equivalent).

(**c**) *Sodium chloride (NaCl), crystal.*—Fluka 71387 (Sigma-Aldrich, or equivalent).

(**d**) *$AgNO_3$.*—Sigma-Aldrich 10220, or equivalent.

D. Solutions

(**a**) *Nitric acid.*—Minimum 65% p.a. (Merck, or equivalent).

(**b**) *Standardized $AgNO_3$ solution, 0.1 M.*—Merck TitriPUR, or equivalent.

(**c**) *NaCl solution, 0.1 M.*—Alfa Aesar (Ward Hill, MA, USA), or equivalent.

E. Preparation of Solutions

(**a**) *Dilute nitric acid solution, 2% (v/v).*—Into a 1000 mL volumetric flask, add about 800 mL water. Carefully pipet 20 mL concentrated nitric acid (65%). Make up to 1000 mL with water. Stopper the volumetric flask and mix well.

Table 2015.08A. Precision of results expressed on reconstituted product

Matrix[a]	Chloride mean, mg/100 g	RSD_r, %[b]	RSD_R, %[c]
NIST SRM 1849a	68.5	0.42	1.59
Child formula powder	46.1	0.26	0.32
Infant elemental powder	36.8	0.10	0.12
Adult nutritional RTF, high protein	38.2	0.64	2.76
Adult nutritional RTF, high fat	30.8	2.77	10.09[d]
Infant formula RTF, milk based (SPIFAN blank milk formula)	20.0	0.22	0.18
Adult nutritional powder milk, protein based	36.4	0.45	0.47
Infant formula powder partially hydrolyzed, milk based	42.4	0.09	0.13
Infant formula powder partially hydrolyzed, soy based	46.2	0.15	0.16
Adult nutritional powder low fat	40.3	0.20	0.18
Child formula powder	38.6	0.67	0.81
Infant elemental powder	39.3	0.21	0.23
Infant formula powder, milk based	46.5	0.88	0.85
Infant formula powder, soy based	56.1	0.29	0.33
Infant formula RTF, milk based (SPIFAN control milk formula)	44.2	0.34	0.24
Adult nutritional RTF, high protein	154.5	0.44	0.40
Adult nutritional RTF, high fat	162.2	1.01	0.78

[a] Samples provided in the SPIFAN I Test Kit. SRM 1849a was reconstituted 10 g to 100 g, and all other powders were reconstituted 25 g to 225 g.

[b] RSD_r = Repeatability RSD.

[c] RSD_R = Reproducibility RSD.

[d] Higher repeatability and intermediate reproducibility were observed in this nonfortified RTF sample compared to the rest of the samples. This is most likely due to sample heterogeneity, since the sample was beyond its expiration date. The sample contents separated even after 15 min shaking prior to opening. These results were not included in the evaluation. Additional high-fat sample was within the SMPR requirements.

Table 2015.08B. Recovery results of spiking experiments for chloride

Matrix	Native chloride, mg/100 g	+ 50% of native value		+ 100% of native value	
		Average, %	RSD, %	Average, %	RSD, %
SRM NIST 1849a	68.5	104.0	2.8	103.0	1.3
Child formula powder	46.1	102.0	2.1	100.0	1.5
Infant elemental powder	36.8	101.0	1.5	101.0	1.5
Infant formula RTF, milk based (SPIFAN blank milk formula)	20.0	103.0	1.2	101.0	1.1
Adult nutritional powder, milk protein based	36.4	101.0	1.2	100.0	0.7
Infant formula powder partially hydrolyzed, milk based	42.4	101.0	1.0	100.0	1.8
Infant formula powder partially hydrolyzed, soy based	46.2	102.0	1.4	100.0	1.5
Adult nutritional powder low fat	40.3	102.0	1.6	101.0	1.8
Child formula powder	38.6	101.0	1.5	100.0	1.3
Infant elemental powder	39.3	100.0	1.9	101.0	1.2
Infant formula powder, milk based	46.5	100.0	0.5	100.0	0.9
Infant formula powder, soy based	56.1	101.0	1.2	100.0	0.4
Infant formula RTF, milk based (SPIFAN control milk formula)	44.2	103.0	3.2	99.0	1.0
Adult nutritional RTF, high protein	154.5	101.0	0.2	100.0	0.5
Adult nutritional RTF, high fat	162.2	102.0	1.6	103.0	1.9

(**b**) *$AgNO_3$ solution, 0.01 M (optional).*—Into a 1000 mL volumetric flask, pipet 100 mL $AgNO_3$ solution, 0.1 M. Make up to the mark with water. Check the titer by titration of 20 mL exactly 0.01 M NaCl solution.

(**c**) *NaCl solution, 0.01 M (optional).*—Into a 1000 mL volumetric flask pipet 100 mL NaCl solution, 0.1 M. Make up to the mark with water.

(**d**) *Standardized $AgNO_3$ solution, 0.1 M.*—If no ready-to-use $AgNO_3$ standard solution is available, weigh 16.9890 ± 0.0005 g $AgNO_3$ previously dried for 2 h at 120 ± 2°C. Dissolve in water and make up to the mark in a 1000 mL volumetric flask. Check the titer by titration of 20 mL exactly 0.1 M NaCl solution.

(**e**) *NaCl solution, 0.1 M.*—If no ready-to-use NaCl standard solution is available, weigh 5.8440 ± 0.0005 g NaCl, previously dried for 2 h at 110 ± 2°C. Dissolve in water and make up to the mark in a 1000 mL volumetric flask.

F. Sample Preparation

Milk product, infant formula, and adult/pediatric nutritional.—Mix well to ensure that sample is homogeneous. Powder samples were reconstituted by dissolving 25 g powder sample in 200 mL warm water (40°C).

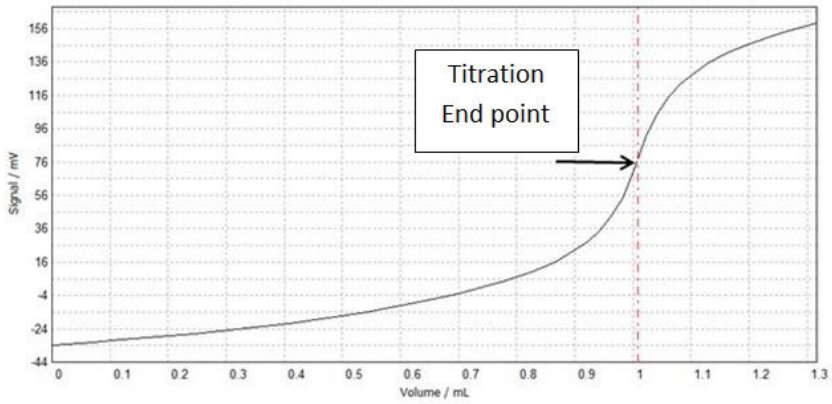
G. Instrument Operating Conditions

Connect the combined silver electrode to the automated titration apparatus according to the manufacturer's instructions. Ensure that the titration vessels are correctly placed on the autosampler and there are enough reagents, both 2% (v/v) nitric acid and 0.1 M $AgNO_3$.

Table 2015.08C. Comparison of SLV data with SMPR

Parameter	SMPR 2014.015	Single-laboratory validation
Matrixes	SLV test matrixes kit (17 samples)	All forms of infant, adult, and/or pediatric formula (powders, RTF liquids, and liquid concentrates)
LOQ, mg/100 g	5 mg/100 g[a]	1.4 mg/100 g[a]
Analytical range, mg/100 g	5–500 mg/100 g[a]	1.4–1060 mg/100 g
Spike recovery, %	95–105	SPIFAN samples (15) were spiked at two levels of chloride on 6 separate days Spike level 1: Average recoveries of 101.6% (range 101–103%) Spike level 2: Average recovery of 100.6% (range 99–103%)
Bias versus SRM	NIST 1849a informational value = 710 mg/100 g	Value found = 685 mg/100 g, recovery = 97.2%, no bias at 95% confidence level
RSD_r (repeatability), %	≤2	Average RSD_r = 0.31% (17 products), range 0.03–1.60%; concentration range evaluated 20–167 mg/100 g RTF
RSD_R (reproducibility), %	≤4	
RSD_{IR} (intermediate reproducibility)	Not assessed	Average RSD_R = 0.54% (17 products); range 0.09–2.77%; concentration range evaluated 20–167 mg/100 g RTF

[a] Concentrations apply to (a) RTF liquids as is; (b) reconstituted powders (25 g into 200 g water); and (c) liquid concentrates diluted 1:1 by weight.



Volume	Increment	Signal	Change	1st deriv.	Time	Temperature
mL	mL	mV	mV	mV/mL	s	oC
0.6405	0.0985	-7.2	7.1	77.21	59	25.0
0.6955	0.0550	-3.2	4.0	92.25	64	25.0
0.7790	0.0835	4.6	7.8	125.68	74	25.0
0.8275	0.0485	10.5	5.9	158.57	81	25.0
0.8655	0.0380	16.1	5.6	201.91	88	25.0
0.9000	0.0345	23.0	6.9	269.55	96	25.0
0.9220	0.0220	28.5	5.5	336.34	102	25.0
0.9420	0.0200	34.8	6.3	404.52	109	25.0
0.9620	0.0200	43.0	8.2	533.58	116	25.0
0.9820	0.0200	54.5	11.5	677.59	124	25.0
1.0020	0.0200	71.2	16.7	779.28	131	25.0
1.007676	NaN	76.6	NaN	781.17	NaN	NaN
1.0220	0.0200	90.2	19.0	755.93	138	25.0
1.0420	0.0200	105.0	14.8	636.30	144	25.0
1.0620	0.0200	115.2	10.2	487.45	149	25.0
1.0820	0.0200	122.8	7.6	376.97	154	25.0
1.1035	0.0215	129.1	6.3	307.43	160	25.0

Figure 2015.08. Typical titration curve obtained from an automatic titrator.

(**a**) *Check and maintenance of the combined silver electrode*.—Rinse electrode with deionized water and wipe before use. Renew the electrolyte periodically per manufacturer's recommendations. If fat sticks to the electrodes during a series of analyses, then eliminate it by briefly immersing the electrode in acetone. Follow manufacturer's recommendations for the storage of electrodes when not in use.

(*Note*: In place of the combined silver electrode, separate silver and reference electrodes may also be used.)

(**b**) *Automated titration*.—Prior to first use, check the system linearity by use of a range of required volumes (e.g., 0.1–15 mL) of 0.1 M NaCl solution. Additionally, prior to each use check the system suitability by preparing three calibration check samples using 5.0 mL 0.1 M NaCl solution.

H. Extraction and Analysis

(**a**) Weigh an appropriate aliquot RTF or reconstituted powder (e.g., 25 g) into a suitable beaker (e.g., 150 mL, manual or semiautomatic procedure) or the autosampler titrator cups (automatic titration). For adult nutritionals with a high chloride content, weigh a smaller test portion, e.g., 5 g reconstituted or RTF product.

(**b**) Add 50 mL 2% (v/v) nitric acid solution as well as a magnetic stirring rod. Place the autosampler cup on a magnetic stirrer and stir until mixed or finely suspended.

(**c**) The pH of the test solution should be below 1.5. In case of doubt, check by means of a pH meter and, if necessary, add more 2% (v/v) nitric acid solution.

(**d**) Under continuous stirring, titrate the sample solution automatically with 0.1 M $AgNO_3$ solution up to the end potential. Record the volume of 0.1 M $AgNO_3$ solution consumed. If performing manual titrations, plot a graph of the variation of potential difference as a function of the quantity of the titrant added, continuing the addition of the titrant beyond the presumed equivalence point. The end point of the titration corresponds to the point at which the potential changes most rapidly (*see* Figure **2015.08**).

(**e**) *Special case: determination of very low amounts of chloride*.—When determining chloride amounts below 20 mg/100 g, for greater accuracy and precision it is preferable to use a 0.01 M $AgNO_3$ solution for the titration. Determine the titer of this solution by means of a 0.01 M NaCl solution.

I. Calculations

Calculate chloride content (w) in mg/100 g RTF or reconstituted sample using the equation:

$$w = \frac{A \times Mw \times Cm \times F \times 100}{m}$$

where A = volume (mL) of 0.1 M or 0.01 M $AgNO_3$ solution used for titration; Mw = atomic weight of chloride (= 35.45 g/mol); *Cm* = exact molar concentration of the $AgNO_3$ solution (0.1000 or 0.0100); m = mass of the test portion, in g; and F = dilution factor for preparation of reconstituted powder or concentrate.

References: *J. AOAC Int*. **98**, 1390(2015)
DOI: 10.5740/jaoacint.15-136

AOAC SMPR 2014.015
J. AOAC Int. **98**, 1079(2015)
DOI: 10.5740/jaoacint.SMPR2014.016

Posted: October 12, 2015

INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Chloride in Infant Formula and Adult/Pediatric Nutritional Formula by Automated Potentiometry: Single-Laboratory Validation, First Action 2015.08

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A direct potentiometric method involving titration against a standard volumetric silver nitrate solution using a silver electrode to detect the end point was evaluated for the determination of chloride in infant formula and adult/pediatric nutritional formula. It was assessed for compliance against AOAC *Standard Method Performance Requirements* (SMPR®) established by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). A single-laboratory validation (SLV) study was conducted as a first step in the process to validate the method. In this SLV, 17 SPIFAN matrixes representing a range of infant formula and adult nutritional products were evaluated for their chloride content. The analytical range was found to be between 1.4 and 1060 mg/100 g reconstituted product or ready-to-feed (RTF) liquid. The LOQ was estimated as 1.4 mg/100 g. Method repeatability was between 0.03 and 1.60% in the range of 20 to 167 mg/100 g RTF, and intermediate precision was between 0.09 and 2.77% in the same range. Recovery values based on spiking experiments at two different levels of chloride ranged from 99.0 to 103% for 15 different SPIFAN products. Evaluation of trueness was performed on National Institute of Standards and Technology Standard Reference Material 1849a (Infant/Adult Nutritional Formula) and showed 97.2% of the theoretical value, with no bias at the 95% confidence level. Based on the results of the SLV, the method met the SMPR and was approved as a First Action method by the AOAC Expert Review Panel on Infant Formula and Adult Nutritionals on March 17, 2015.

Received May 26, 2015. Accepted by SG July 2, 2015.
The method was approved by the Expert Review Panel on Infant Formula and Adult Nutritionals as First Action.

The Expert Review Panel on Infant Formula and Adult Nutritionals invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

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DOI: 10.5740/jaoacint.15-136

A potentiometric method was evaluated to establish an international consensus method for the determination of chloride in infant formula and adult/pediatric nutritional formula. Standard Method Performance Requirements (SMPR®) for chloride were approved by the AOAC Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN) and are described in AOAC SMPR 2014.015 (1). The single-laboratory validation (SLV) was conducted on 17 SPIFAN matrixes and a Standard Reference Material [SRM; National Institute of Standards and Technology (NIST) 1849a Infant Formula/Adult Nutritional Formula]. LOQ, precision, and accuracy were assessed in this study.

On March 17, 2015, an AOAC Expert Review Panel (ERP) on Infant Formula and Adult Nutritionals reviewed the SLV study on the potentiometric method for determination of chloride and adopted the method as AOAC *Official Method*℠ **2015.08**. The next step in the process will be to have a multiple laboratory study performed using SPIFAN kits, after which the ERP may recommend the method to the AOAC Official Methods Board for Final Action Status (2).

AOAC Official Method 2015.08
Determination of Chloride in Infant Formula and Adult/Pediatric Nutritional Formula
Potentiometry
First Action 2015

[Applicable to the determination of chloride in ready-to-feed (RTF) liquid concentrate and powder products from levels of 1.4 to 1060 mg/100 g reconstituted product or RTF liquids.]

See Tables **2015.08 A–C** for the results of the SLV study supporting acceptance of the method. The method was evaluated against Standard Method Performance Requirements AOAC SMPR 2014.015 (1).

A. Principle

Reconstitute powder samples by dissolving 25 g powder sample in 200 g warm water (40°C). Add 50 mL of 2% (v/v) nitric acid solution. Stir with a magnetic stirrer until mixed or finely suspended. Ensure pH is <1.5. Titrate potentiometrically against standardized silver nitrate ($AgNO_3$) solution, 0.1 M, using a silver electrode to detect the end point.

B. Apparatus

Common laboratory equipment and, in particular, the following:

Table 2015.08A. Precision of results expressed on reconstituted product

Matrix[a]	Chloride mean, mg/100 g	RSD_r, %[b]	RSD_R, %[c]
NIST SRM 1849a	68.5	0.42	1.59
Child formula powder	46.1	0.26	0.32
Infant elemental powder	36.8	0.10	0.12
Adult nutritional RTF, high protein	38.2	0.64	2.76
Adult nutritional RTF, high fat	30.8	2.77	10.09[d]
Infant formula RTF, milk based (SPIFAN blank milk formula)	20.0	0.22	0.18
Adult nutritional powder milk, protein based	36.4	0.45	0.47
Infant formula powder partially hydrolyzed, milk based	42.4	0.09	0.13
Infant formula powder partially hydrolyzed, soy based	46.2	0.15	0.16
Adult nutritional powder low fat	40.3	0.20	0.18
Child formula powder	38.6	0.67	0.81
Infant elemental powder	39.3	0.21	0.23
Infant formula powder, milk based	46.5	0.88	0.85
Infant formula powder, soy based	56.1	0.29	0.33
Infant formula RTF, milk based (SPIFAN control milk formula)	44.2	0.34	0.24
Adult nutritional RTF, high protein	154.5	0.44	0.40
Adult nutritional RTF, high fat	162.2	1.01	0.78

[a] Samples provided in the SPIFAN I Test Kit. SRM 1849a was reconstituted 10 g to 100 g, and all other powders were reconstituted 25 g to 225 g.

[b] RSD_r = Repeatability RSD.

[c] RSD_R = Reproducibility RSD.

[d] Higher repeatability and intermediate reproducibility were observed in this nonfortified RTF sample compared to the rest of the samples. This is most likely due to sample heterogeneity, since the sample was beyond its expiration date. The sample contents separated even after 15 min shaking prior to opening. These results were not included in the evaluation. Additional high-fat sample was within the SMPR requirements.

Table 2015.08B. Recovery results of spiking experiments for chloride

		+ 50% of native value		+ 100% of native value	
Matrix	Native chloride, mg/100 g	Average, %	RSD, %	Average, %	RSD, %
SRM NIST 1849a	68.5	104.0	2.8	103.0	1.3
Child formula powder	46.1	102.0	2.1	100.0	1.5
Infant elemental powder	36.8	101.0	1.5	101.0	1.5
Infant formula RTF, milk based (SPIFAN blank milk formula)	20.0	103.0	1.2	101.0	1.1
Adult nutritional powder, milk protein based	36.4	101.0	1.2	100.0	0.7
Infant formula powder partially hydrolyzed, milk based	42.4	101.0	1.0	100.0	1.8
Infant formula powder partially hydrolyzed, soy based	46.2	102.0	1.4	100.0	1.5
Adult nutritional powder low fat	40.3	102.0	1.6	101.0	1.8
Child formula powder	38.6	101.0	1.5	100.0	1.3
Infant elemental powder	39.3	100.0	1.9	101.0	1.2
Infant formula powder, milk based	46.5	100.0	0.5	100.0	0.9
Infant formula powder, soy based	56.1	101.0	1.2	100.0	0.4
Infant formula RTF, milk based (SPIFAN control milk formula)	44.2	103.0	3.2	99.0	1.0
Adult nutritional RTF, high protein	154.5	101.0	0.2	100.0	0.5
Adult nutritional RTF, high fat	162.2	102.0	1.6	103.0	1.9

Table 2015.08C. Comparison of SLV data with SMPR requirements

Parameter	SMPR 2014.015	Single-laboratory validation
Matrixes	SLV test matrixes kit (17 samples)	All forms of infant, adult, and/or pediatric formula (powders, RTF liquids, and liquid concentrates)
LOQ, mg/100 g	5 mg/100 g[a]	1.4 mg/100 g[a]
Analytical range, mg/100 g	5–500 mg/100 g[a]	1.4–1060 mg/100 g
Spike recovery, %	95–105	SPIFAN samples (15) were spiked at two levels of chloride on 6 separate days Spike level 1: average recoveries of 101.6% (range 101–103%) Spike level 2: average recovery of 100.6% (range 99–103%)
Bias versus SRM	NIST 1849a informational value = 710 mg/100 g	Value found = 685 mg/100 g, recovery = 97.2%, no bias at 95% confidence level
RSD_r (repeatability), %	≤2	Average RSD_r = 0.31% (17 products), range 0.03–1.60%; concentration range evaluated 20–167 mg/100 g RTF
RSD_R (reproducibility), %	≤4	
RSD_{IR} (intermediate reproducibility)	Not assessed	Average RSD_R = 0.54% (17 products); range 0.09–2.77%; concentration range evaluated 20–167 mg/100 g RTF

[a] Concentrations apply to: (a) RTF liquids as is; (b) reconstituted powders (25 g into 200 g water); and (c) liquid concentrates diluted 1:1 by weight.

(a) *Analytical balance.*—Precision 0.1 mg.

(b) *Class A volumetric flasks.*—100 and 1000 mL.

(c) *pH meter/mV meter with a scale covering ±700 mV, and buret, 20 or 25 mL.*—Mettler-Toledo (Columbus, OH) or equivalent.

(d) *Automatic titrator.*—Autosampler, motorized piston buret, with remote-control dispensing and filling (Mettler Toledo T50 Rondo Tower autosampler, Mettler LabX 3.1 software, or equivalent).

(e) *Combined ring silver electrode.*—e.g., Mettler Toledo DM 141 or DMi145-SC, or equivalent; alternatively, a silver electrode with reference electrode can be used.

(f) *Magnetic stirrer.*—Heidolph MR 3000 or equivalent (Sigma-Aldrich, St. Louis, MO).

(g) *Water bath.*—Capable of warming water to 40°C.

(h) *Laboratory oven.*—Capable of heating to 120°C.

(i) *Pipets (1, 20, 50, and 100 mL).*—Class A glass volumetric or automatic (Eppendorf or equivalent).

(j) *Buret.*—10 mL.

C. Chemicals and Reagents

(a) *Acetone.*—p.a. (Merck, Darmstadt, Germany, or equivalent).

(b) *Water, purified.*—Greater than 18MΩ (EMD Millipore Corp., Billerica, MA, or equivalent).

(c) *Sodium chloride (NaCl), crystal.*—Fluka 71387 (Sigma-Aldrich, or equivalent).

(d) *$AgNO_3$.*—Sigma-Aldrich 10220, or equivalent.

D. Solutions

(a) *Nitric acid.*—Minimum 65% p.a. (Merck, or equivalent).

(b) *Standardized $AgNO_3$ solution, 0.1 M.*—Merck TitriPUR, or equivalent.

(c) *NaCl solution, 0.1 M.*—Alfa Aesar (Ward Hill, MA), or equivalent.

E. Preparation of Solutions

(a) *Dilute nitric acid solution, 2% (v/v).*—Into a 1000 mL volumetric flask, add about 800 mL water. Carefully pipette 20 mL concentrated nitric acid (65%). Make up to 1000 mL with water. Stopper the volumetric flask and mix well.

(b) *$AgNO_3$ solution, 0.01 M (optional).*—Into a 1000 mL volumetric flask, pipette 100 mL $AgNO_3$ solution, 0.1 M. Make up to the mark with water. Check the titer by titration of 20 mL exactly 0.01 M NaCl solution.

(c) *NaCl solution, 0.01 M (optional).*—Into a 1000 mL volumetric flask pipette 100 mL NaCl solution, 0.1 M. Make up to the mark with water.

(d) *Standardized $AgNO_3$ solution, 0.1 M.*—If no ready-to-use $AgNO_3$ standard solution is available, weigh 16.9890 ± 0.0005 g $AgNO_3$ previously dried for 2 h at 120 ± 2°C. Dissolve in water and make up to the mark in a 1000 mL volumetric flask. Check the titer by titration of 20 mL exactly 0.1 M NaCl solution.

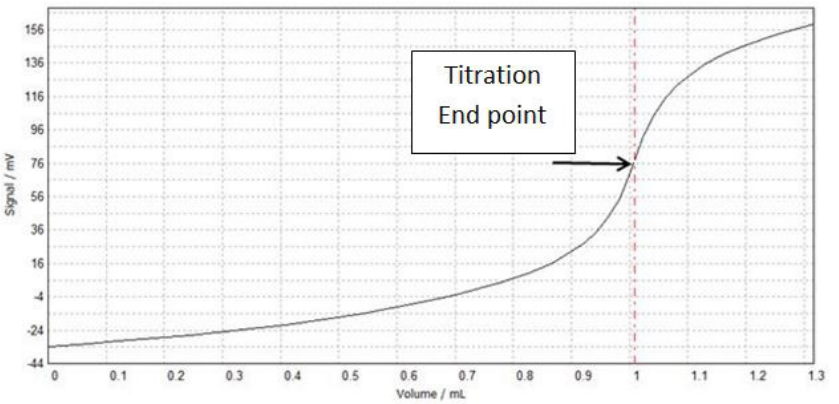
(e) *NaCl solution, 0.1 M.*—If no ready-to-use NaCl standard solution is available, weigh 5.8440 ± 0.0005 g NaCl, previously dried for 2 h at 110 ± 2°C. Dissolve in water and make up to the mark in a 1000 mL volumetric flask.

F. Sample Preparation

Milk product, infant formula, and adult/pediatric nutritional.—Mix well to ensure that sample is homogeneous. Powder samples were reconstituted by dissolving 25 g powder sample in 200 mL warm water (40°C).

G. Instrument Operating Conditions

Connect the combined silver electrode to the automated titration apparatus according to the manufacturer's instructions. Ensure that the titration vessels are correctly placed on the autosampler and there are enough reagents, both 2% (v/v) nitric acid and 0.1 M $AgNO_3$.



Volume mL	Increment mL	Signal mV	Change mV	1st deriv. mV/mL	Time s	Temperature oC
0.6405	0.0985	-7.2	7.1	77.21	59	25.0
0.6955	0.0550	-3.2	4.0	92.25	64	25.0
0.7790	0.0835	4.6	7.8	125.68	74	25.0
0.8275	0.0485	10.5	5.9	158.57	81	25.0
0.8655	0.0380	16.1	5.6	201.91	88	25.0
0.9000	0.0345	23.0	6.9	269.55	96	25.0
0.9220	0.0220	28.5	5.5	336.34	102	25.0
0.9420	0.0200	34.8	6.3	404.52	109	25.0
0.9620	0.0200	43.0	8.2	533.58	116	25.0
0.9820	0.0200	54.5	11.5	677.59	124	25.0
1.0020	0.0200	71.2	16.7	779.28	131	25.0
1.007676	NaN	76.6	NaN	781.17	NaN	NaN
1.0220	0.0200	90.2	19.0	755.93	138	25.0
1.0420	0.0200	105.0	14.8	636.30	144	25.0
1.0620	0.0200	115.2	10.2	487.45	149	25.0
1.0820	0.0200	122.8	7.6	376.97	154	25.0
1.1035	0.0215	129.1	6.3	307.43	160	25.0

Figure 2015.08. Typical titration curve obtained from an automatic titrator.

(a) *Check and maintenance of the combined silver electrode*.—Rinse electrode with deionized water and wipe before use. Renew the electrolyte periodically per manufacturer's recommendations. If fat sticks to the electrodes during a series of analyses, then eliminate it by briefly immersing the electrode in acetone. Follow manufacturer's recommendations for the storage of electrodes when not in use.

(*Note*: In place of the combined silver electrode, separate silver and reference electrodes may also be used.)

(b) *Automated titration*.—Prior to first use, check the system linearity by use of a range of required volumes (e.g., 0.1–15 mL) of 0.1 M NaCl solution. Additionally, prior to each use check the system suitability by preparing three calibration check samples using 5.0 mL 0.1 M NaCl solution.

H. Extraction and Analysis

(a) Weigh an appropriate aliquot RTF or reconstituted powder (e.g., 25 g) into a suitable beaker (e.g., 150 mL, manual or semiautomatic procedure) or the autosampler titrator cups (automatic titration). For adult nutritionals with a high chloride content, weigh a smaller test portion, e.g., 5 g reconstituted or RTF product.

(b) Add 50 mL 2% (v/v) nitric acid solution as well as a magnetic stirring rod. Place the autosampler cup on a magnetic stirrer and stir until mixed or finely suspended.

(c) The pH of the test solution should be below 1.5. In case of doubt, check by means of a pH meter and, if necessary, add more 2% (v/v) nitric acid solution.

(d) Under continuous stirring, titrate the sample solution automatically with 0.1 M $AgNO_3$ solution up to the end potential. Record the volume of 0.1 M $AgNO_3$ solution consumed. If performing manual titrations, plot a graph of the variation of potential difference as a function of the quantity of the titrant added, continuing the addition of the titrant beyond the presumed equivalence point. The end point of the titration corresponds to the point at which the potential changes most rapidly (*see* Figure **2015.08**).

(e) *Special case: determination of very low amounts of chloride*.—When determining chloride amounts below 20 mg/100 g, for greater accuracy and precision it is preferable to use a 0.01 M $AgNO_3$ solution for the titration. Determine the titer of this solution by means of a 0.01 M NaCl solution.

I. Calculations

Calculate chloride content (w) in mg/100 g RTF or reconstituted sample using the equation:

$$w = \frac{A \times Mw \times Cm \times F \times 100}{m}$$

where A = volume (mL) of 0.1 M or 0.01 M $AgNO_3$ solution used for titration; Mw = atomic weight of chloride (= 35.45 g/mol); *Cm* = exact molar concentration of the $AgNO_3$ solution (0.1000 or 0.0100); m = mass of the test portion, in g; and F = dilution factor for preparation of reconstituted powder or concentrate.

Results

The validation study was conducted in accordance with the SPIFAN SLV guidelines (3).

The analytical range for SPIFAN samples was found to be between 1.4 and 1060 mg/100 g reconstituted product or RTF. The LOQ was estimated as 1.4 mg/100 g. Results of precision studies for chloride expressed on reconstituted products are presented in Table **2015.08A**. RSD_r ranged from 0.03 to 1.60% in the range from 20 to 167 mg/100 g RTF, and RSD_R ranged from 0.09 to 2.77% over the same range, thus fulfilling the ≤ 2 and 4% performance requirements, respectively. All samples met the intermediate reproducibility (RSD_R) performance requirement except for one RTF sample with high fat content, which gave a precision value of 10.09%. This RTF sample was problematic due to heterogeneity and separation since it was being used beyond its recommended shelf life.

Accuracy was proven by analyzing SRM 1849a in duplicate on 6 different days and comparing the overall mean to the SRM informational value (not certified; Table **2015.08B**). The overall mean was 68.5 mg/100 g for the reconstituted product with an RSD_R of 1.59%, equivalent to a recovery of 97.2%. Spike recovery was performed on 15 different SPIFAN products (powder and liquid infant formula and adult nutritionals). The reconstituted powders and RTF products were spiked at two different levels (50 and 100% of native chloride content) and analyzed in duplicate on 6 different days (Table **2015.08B**). Spike recovery (99–104%) was within the tolerance (95–105%) defined in the SMPR.

All results compared to SMPR values are shown in Table **2015.08C**. All results were within the target values defined in AOAC SMPR 2014.015.

Conclusions

The data presented in this paper were submitted to the AOAC ERP for review at the AOAC INTERNATIONAL Mid-Year Meeting held on March 17, 2015. The ERP determined that the data presented were in accordance with SMPR 2014.015 approved by SPIFAN, and the method was granted First Action status.

References

(1) AOAC SMPR 2014.015, *J. AOAC Int*. **98**, 1079. http://dx.doi.org/10.5740/jaoacint.SMPR2014.016

(2) Sullivan, D. (2012) *J. AOAC Int*. **95**, 278–297. http://dx.doi.org/10.5740/jaoacint.Sullivan_Intro

(3) *Official Methods of Analysis* (2012) 19th Ed., AOAC INTERNATIONAL, Rockville, MD, Appendix L: AOAC Recommended Guidelines for Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Single-Laboratory Validation, http://www.eoma.aoac.org

AOAC Official Method 2015.09
Trans Vitamin K_1
in Infant, Pediatric, and Adult Nutritionals
HPLC with Fluorescence Detection
First Action 2015

(Applicable to the determination of *trans* vitamin K_1 in infant, pediatric, and adult nutritional formulas.)

Caution: Refer to Material Safely Data Sheets (MSDS) of chemicals prior to use and use the suggested personal protective equipment. Zinc powder should be handled in a fume hood; it is self-heating and may catch fire. Zinc powder should not be allowed to come into contact with water, which may release flammable gases that may spontaneously ignite.

See Tables **2015.09A** and **B** for infant, pediatric, and nutritional matrixes for which the method has been validated; and for study results supporting acceptance of the method.

A. Principle

This normal-phase (NP) HPLC method with postcolumn reduction and fluorescence detection allows for the quantitative determination of *trans* vitamin K_1 in infant, pediatric, and adult nutritionals. Vitamin K_1 is extracted from products with iso-octane after precipitation of proteins and release of lipids with methanol. Prepared samples are injected onto a silica HPLC column where *cis* and *trans* vitamin K_1 are separated with an iso-octane–isopropanol mobile phase. The column eluent is mixed with a dilute ethanolic solution of zinc chloride, sodium acetate, and acetic acid, and vitamin K_1 is reduced to a fluorescent derivative in a zinc reactor column. The resulting fluorescent compound is then detected by fluorescence at an excitation wavelength of 245 nm and an emission wavelength of 440 nm.

B. Apparatus and Materials

(a) *HPLC system*.—Two isocratic pumps; autosampler capable of injecting 20 µL; fluorescence detector; high-pressure mixing tee; and postcolumn reactor column 20 × 4 mm stainless steel (Waters, Milford, MA, USA; Part No. WAT084550 or equivalent). The system should be configured as shown in Figure **2015.09A**.

(b) *Analytical column*.—Silica 150 × 3.0 mm, 3 µm, 60 Å, or equivalent.

(c) *Analytical balance*.—Capable of weighing to the nearest 0.00001 g.

(d) *Beakers*.—Glass, assorted sizes.

(e) *Centrifuge*.

(f) *Centrifuge tubes and caps*.—50 mL glass tubes with Teflon-lined caps.

(g) *Cylinders*.—Graduated, glass, assorted sizes.

(h) *Gas regulator*.—Helium.

(i) *Helium sparge*.—Tubing and filtering assembly.

(j) *Magnetic stirrer*.—Variomag Telesystem (Daytona Beach, FL, USA) HP 60 S (Part No. HP 46040U) with tube rack (Part No. HP 86029) and egg-shaped Teflon stirring bars (Part No. PI20110) or equivalent.

(k) *Pipet*.—Disposable glass, Pasteur.

(l) *Pipet*.—Mechanical, variable volume, 0.5–5 mL and 10–100 µL.

(m) *Pipet*.—Repeating 5 and 25 mL or equivalent.

(n) *Volumetric flasks*.—Glass, Class A, assorted sizes.

(o) *Volumetric pipets*.—Glass, Class A, assorted sizes.

(p) *Vortex mixers*.

(q) *Yellow lights or yellow shields with cutoff of at least 440 nm*.

C. Reagents

(a) *Acetic acid*.—Glacial, ACS.

(b) *Helium*.—Zero grade or equivalent.

(c) *Iso-octane (2,2,4-trimethylpentane)*.—HPLC grade.

Table 2015.09A. *Trans* vitamin K_1 SLV data—precision

Sample type	No. of replicates (duplicates on multiple days)	Mean, µg/100 g RTF	SD_r	RSD_r, %	SD_{IP}	RSD_{IP}, %
Child formula powder, placebo	6	2.03	0.040	2.0	0.045	2.2
Infant elemental powder, placebo	6	2.03	0.020	1.0	0.025	1.2
Adult nutritional RTF high protein, placebo	6	3.47	0.021	0.6	0.040	1.2
Adult nutritional RTF high fat, placebo	6	3.07	0.034	1.1	0.034	1.1
Infant formula RTF milk based, placebo	6	2.16	0.038	1.8	0.038	1.8
SRM 1849a	12	1.11[a]	0.022	2.0	0.025	2.3
Adult nutritional powder milk protein based	12	3.26	0.097	3.0	0.142	4.4
Infant formula powder partially hydrolyzed milk based	12	7.69	0.123	1.6	0.170	2.2
Infant formula powder partially hydrolyzed soy based	12	8.99	0.141	1.6	0.226	2.5
Adult nutritional powder low fat	12	2.92	0.102	3.5	0.102	3.5
Child formula powder	12	2.66	0.049	1.8	0.050	1.9
Infant elemental powder	12	7.57	0.129	1.7	0.451	6.0
Infant formula powder milk based	12	6.09	0.105	1.7	0.131	2.2
Infant formula powder soy based	12	6.26	0.105	1.7	0.211	3.4
Infant formula RTF milk based	12	9.01	0.168	1.9	0.189	2.1
Adult nutritional RTF high protein	16	9.10	0.283	3.1	0.299	3.3
Adult nutritional RTF high fat	12	10.7	0.120	1.1	0.179	1.7

[a] Results reported as mg/kg powder.

Table 2015.09B. *Trans* vitamin K_1 SLV data–accuracy

Sample type	No. of replicates (duplicates on multiple days)	Native level, µg/100 g RTF	Spike level: 100% Recovery, %	100% RSD, %	50% Recovery, %	50% RSD, %
Child formula powder	6	2.66	98.2	5.9	96.2	7.1
Infant elemental powder	6	7.57	93.2	7.6	94.0	2.6
SRM 1849a	6	1.11[a]	104	2.9	95.5	1.8
Adult nutritional powder milk protein based	6	3.26	96.4	2.0	95.1	1.8
Infant formula powder partially hydrolyzed milk based	6	7.69	96.6	3.6	91.9	3.0
Infant formula powder partially hydrolyzed soy based	6	8.99	97.9	1.0	96.1	2.6
Adult nutritional powder low fat	6	2.92	98.0	2.5	95.2	2.6
Infant formula powder milk based	6	6.09	97.6	1.0	102	2.3
Infant formula powder soy based	6	6.26	97.9	1.7	102	0.3
Infant formula RTF milk based	6	9.01	100	1.3	104	0.4
Adult nutritional RTF high protein	6	9.10	96.7	2.7	106	0.7
Adult nutritional RTF high fat	6	10.7	98.2	1.2	93.8	4.0

[a] Results reported as mg/kg powder.

(d) *Isopropanol (isopropyl alcohol)*.—HPLC grade.

(e) *Methanol*.—HPLC grade.

(f) *Phytonadione/phylloquinone (vitamin K_1)*.—U.S. Pharmacopeia Reference, Official Lot. Store per label instructions.

(g) *Laboratory water*.

(h) *Reagent alcohol*.—ACS.

(i) *Sodium acetate anhydrous*.—ACS.

(j) *Zinc*.—Sigma-Aldrich (St. Louis, MO, USA) Part No. 324930, <150 µm, 99.995% or equivalent.

(k) *Zinc chloride*.—ACS.

D. Standard and Solution Preparation

Note: Since vitamin K_1 is light-sensitive, all standards must be prepared, handled, and stored in the dark or under yellow shielded lighting [*see* **B(q)**] unless otherwise stated. If the standards must be transported through or into an area without yellow shielded lighting, they must be wrapped tightly in foil. All standard solutions must be prepared using Class A volumetric glassware.

(a) *Mobile phase*.—Add about 900 mL iso-octane to a 1000 mL volumetric flask. Add 0.3–0.4 mL isopropanol to the volumetric flask and dilute to volume with iso-octane. (*Note*: The isopropanol concentration in the mobile phase can be adjusted slightly until baseline resolution of *cis* and *trans* vitamin K_1 from other peaks present in some samples is achieved. *See* Figures **2015.09B–D**).

(b) *Postcolumn electrolyte solution*.—Transfer 0.25 (±0.02) g zinc chloride and 0.10 (±0.05) g sodium acetate anhydrous to a 1000 mL volumetric flask with reagent alcohol. Add 75 (±5) µL concentrated acetic acid and dilute to volume with reagent alcohol. Add a stir bar and stir solution for about 30 min or until solution is clear and all salts are dissolved.

(c) *Vitamin K_1 (phytonadione) stock standard solution*.—Accurately weigh to 0.00001 g about 0.05500 g vitamin K_1 (phytonadione) into a 250 mL volumetric flask. Dissolve oil and dilute to volume with iso-octane. Store refrigerated in a tightly stoppered container protected from light. Expiration 6 months.

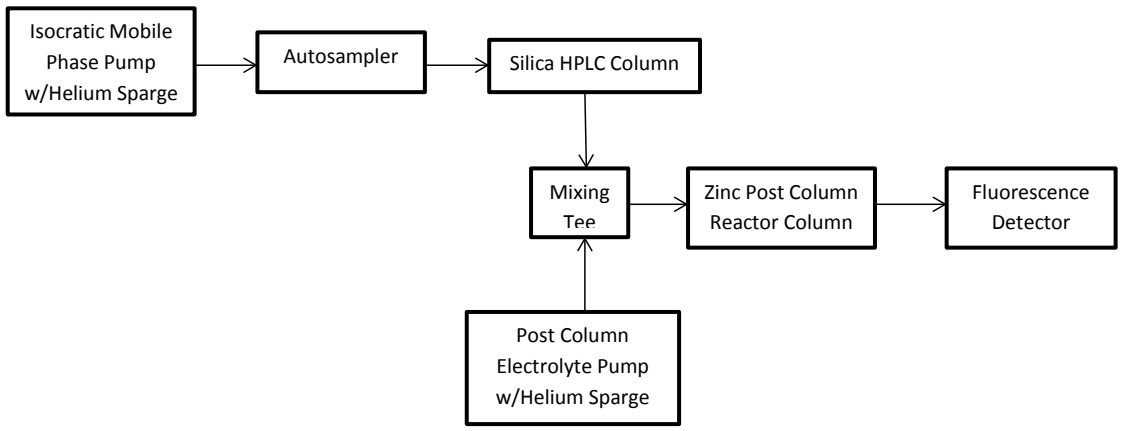


Figure 2015.09A. Vitamin K system configuration.

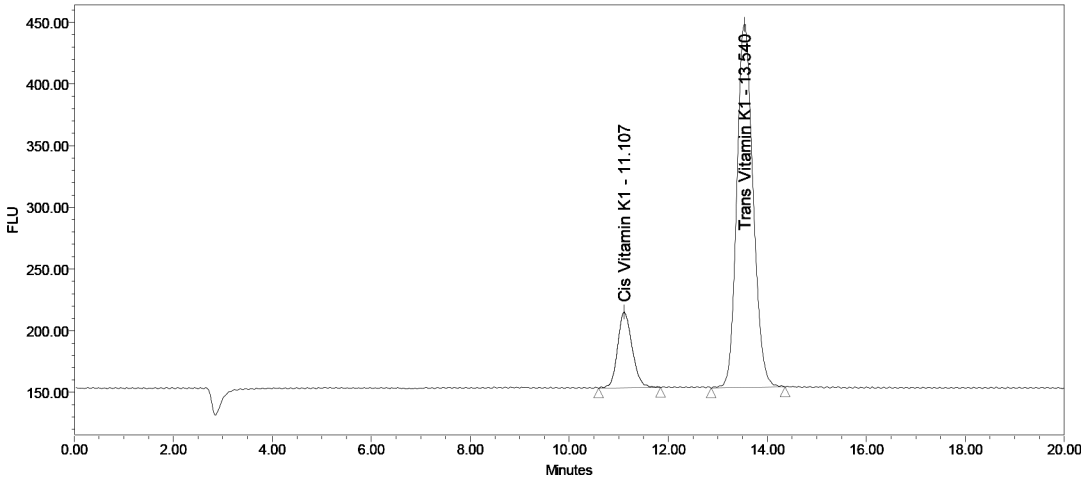


Figure 2015.09B. Chromatogram of vitamin K_1 standard.

(d) *Vitamin K_1 (phytonadione) intermediate I standard solution.*—Dilute 1.0 mL vitamin K_1 stock standard to 100 mL with iso-octane. Prepare fresh each time new working standards are made.

(e) *Vitamin K_1 (phytonadione) intermediate II standard solution.*—Dilute 10.0 mL vitamin K_1 intermediate I standard to 50 mL with iso-octane. Prepare fresh each time new working standards are made.

(f) *Vitamin K_1 (phytonadione) working standard solutions.*—Dilute 1.0, 3.0, 6.0, 10.0, and 20.0 mL intermediate II standard to 100 mL with iso-octane. Store refrigerated in tightly stoppered containers protected from light. Expiration 3 months.

(*Note*: Transfer working standards to autosampler vials with Pasteur pipets or equivalent glass. Do not pour the standards from the volumetric flasks into vials.)

E. Procedure

[(*Note*: Because vitamin K_1 is light-sensitive, all samples must be prepared, handled, and stored in the dark or under yellow-shielded lighting, **B(q)** unless otherwise stated. If the samples must be transported through or into an area without yellow-shielded lighting, they must be wrapped tightly in foil.)]

(a) *Sample preparation.*—(*1*) Accurately weigh to 0.0001 g, up to 0.5 g homogeneous powder or up to 4 g of ready-to-feed (RTF) liquids or nonhomogeneous powders diluted to RTF concentrations into 50 mL centrifuge tubes. To powders weighed directly into the 50 mL centrifuge tubes, add 4 mL water and mix well. To liquids with sample weights that are less than 4 g, add enough water to the tubes so that the sample weight plus the amount of added water equals about 4 and mix well.

(*2*) Add 25 (±2.0) mL methanol to each sample just prior to vortexing or stirring. Methanol should not be added to more than two samples consecutively without vortexing or stirring. Cap each centrifuge tube. Vortex each sample at high speed for at least 30 s, and allow samples to sit undisturbed for at least 10 min, but no more than 40 min, after vortexing with methanol, or add a magnetic stir bar to each sample, place each capped sample onto a magnetic stir plate, and stir each sample for at least 10 min, but not more than 40 min, at a spin rate that causes a vortex.

(*3*) Add 10 (±0.05) mL iso-octane to each sample with a volumetric pipet and cap tubes. Iso-octane can be added to all samples before vortexing or stirring any of the samples. Vortex each sample for at least 45 s or stir each sample for at least 45 s at a spin rate that causes a vortex to form within the sample.

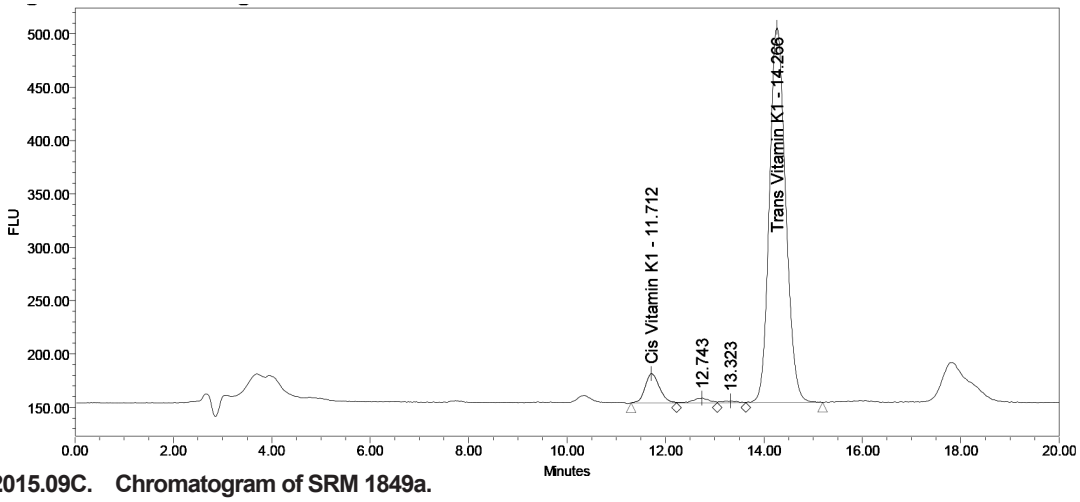


Figure 2015.09C. Chromatogram of SRM 1849a.

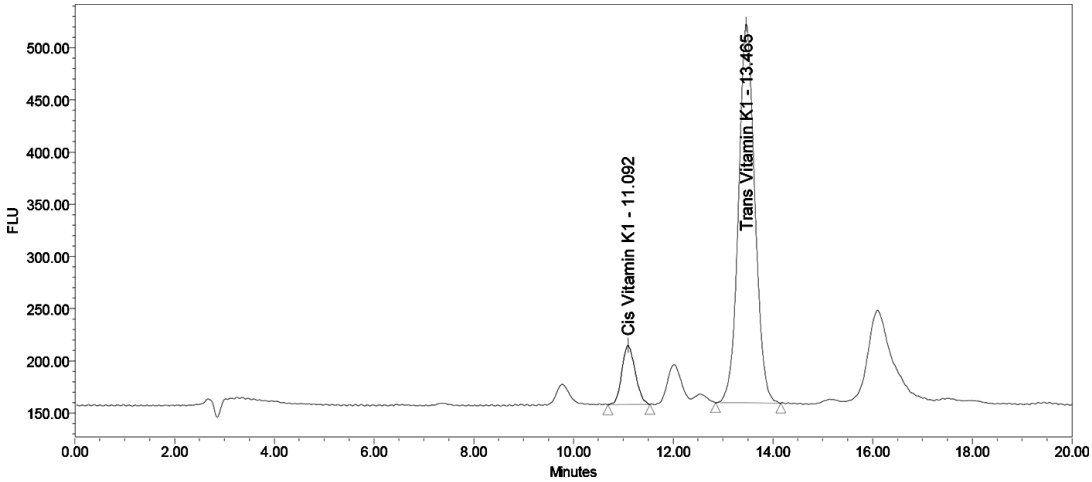


Figure 2015.09D. Chromatogram of an adult nutritional.

(*4*) Add 5 (±1) mL laboratory water to each sample and cap tubes. Laboratory water can be added to all the samples prior to vortexing or stirring. Vortex or shake each sample for at least 20 s or stir each sample for at least 20 s at a spin rate that causes a vortex to form within the sample.

(*5*) Centrifuge the samples until a clean separation of the iso-octane and laboratory water–methanol layers results. The iso-octane layer should be a clear layer at the top of the centrifuge tube, and the laboratory water–methanol layer should be a cloudy layer below the iso-octane layer. (A good separation of solvent layers can usually be achieved by centrifuging samples for approximately 10 min at 800 relative centrifugal force.)

(*6*) Remove samples from the centrifuge and inspect the samples to verify that the iso-octane and laboratory water–methanol layers are separated. With a glass pipet, carefully rinse down the upper walls of the centrifuge tube with a portion of the iso-octane layer. If the layers become mixed together, centrifuge the sample again. Pipette a portion of the clear iso-octane layer into a labeled autosampler vial and cap the vial.

(b) *HPLC analysis*.—(*1*) *Instrumental operating conditions*.—(*a*) HPLC analytical column pump flow rate, 0.4 mL/min. (*b*) Postcolumn flow rate, 0.4 mL/min. (*c*) Injection volume, 20 µL. (*d*) Run time, 20 min. (*e*) Fluorescence excitation and emission, 245 and 440 nm, respectively.

(*2*) *Instrument startup*.—The system should be configured as shown in Figure **2015.09B**.

If necessary, remove used zinc and repack the postcolumn reactor column with fresh zinc. The zinc reactor column should be repacked whenever the S/N in the lowest standard is too high to accurately integrate the vitamin K_1 peak, when peak responses from injections of the same standard drop by more than 7% and the drop cannot be attributed to other system components, or when the system back pressure through the zinc reactor increases significantly and vitamin K_1 peak widths begin to increase. To repack the zinc reactor column, remove the hex nuts and retainers from both ends of the column and force the used zinc out of the column with a thin wire or similar apparatus. Flush the zinc reactor column with ethanol to remove residual zinc. Replace the hex nut and retainer on one end of the zinc reactor column. Carefully transfer a small amount of zinc powder to the reactor column with a spatula, and press down on the zinc in the column with an old HPLC piston or similar apparatus to pack it tightly. Continue adding zinc and pressing it down until the level of zinc is even with the top of the column. After the reactor column is full, replace the second retainer and hex nut. The more tightly zinc is packed into the reactor column, the more symmetrical the vitamin K_1 peaks will be. Degas the mobile phase and postcolumn electrolyte solutions by bubbling helium through them at a flow rate just fast enough to cause small ripples on the surface of the mobile phase and postcolumn solutions. To maximize the life of the zinc reactor column, degas the mobile phase and postcolumn electrolyte solution for at least 30 min before connecting the zinc reactor column or do not pump mobile phase and postcolumn electrolyte solutions until at least 30 min after degassing begins. Once the mobile phase and postcolumn electrolyte solutions have been degassed, allow the column and postcolumn reactor to equilibrate with mobile phase flowing at 0.4 mL/min and postcolumn electrolyte solution flowing at 0.4 mL/min for at least 30 min prior to the first injection if the zinc reactor has been used for previous analyses or several hours if the zinc postcolumn reactor has been freshly packed. Once the mobile phase and postcolumn solutions have been degassed, reduce the helium flow rate so that only a small stream of helium bubbles are visible in the mobile phase and postcolumn solutions and there is minimal disturbance to the surface of these solutions. Bubble helium very slowly through the mobile phase and postcolumn electrolyte solutions continuously throughout the entire run. Once the run has started, do not adjust the helium flow rate. Allow the fluorescence detector lamp to warm up 30 min prior to the first injection. (*Note*: When the mobile phase and postcolumn electrolyte solution are continuously sparged with helium throughout a run, it is not necessary to pack the postcolumn reactor with zinc at the beginning of every run. It should be possible to analyze hundreds of samples before the zinc reactor column must be repacked.)

(*3*) *HPLC of standards and samples*.—Inject the most concentrated standard (approximately 80 µg/L) onto the column and observe the response on the fluorescence detector. If necessary, adjust the detector gain and sensitivity settings so that the standard response is within the range of the detector. Once the detector settings have been determined, inject the most concentrated standard 3–4 times and note the peak areas. If the system is equilibrated, the RSD of the standard peak areas should be ≤2%, and the peak areas should not steadily increase or decrease by more than 4% from the

first injection to the third or fourth injection. If the RSD is >2%, locate the source of the imprecision and correct it before beginning the sample analysis. If peak areas steadily increase or decrease by more than 4%, the system is not equilibrated and must be allowed to equilibrate longer. Once the system has reached equilibrium and the RSD is ≤2%, inject a set of standards, unknown samples, and another set of standards. Every set of unknown samples must be bracketed by standards.

(*4*) *Instrument shut down*.—After analyzing a set of samples, simultaneously turn off the flow on the mobile phase and postcolumn electrolyte solution pumps. Remove the helium sparge lines from the mobile phase and postcolumn electrolyte solution and turn off the helium. Turn off the fluorescence detector lamp.

F. Calculations

The vitamin K_1 concentrations of samples analyzed on the HPLC system are determined by comparison of peak areas from samples of known weight with the peak areas of standards of known concentration. Because the *cis* and *trans* vitamin K_1 retention times may shift slightly during a run, peak areas must be used to quantitate *trans* vitamin K_1.

(a) Calculation of the standard concentrations:

$$C_s = \frac{W \times (V_1 \times V_2 \times V_3) \times 1{,}000{,}000{,}000}{(D_1 \times D_2 \times D_3 \times D_4)}$$

where C_s is the working standard concentration in µg/L; W is the weight of standard in g; V_1, V_2, and V_3 are the aliquots of stock, intermediate I, and intermediate standard II solutions, respectively, in mL; 1,000,000,000 is the conversion factor from g/mL to mcg/L; and D_1, D_2, D_3, and D_4 are the dilution volumes of the stock, intermediate I, intermediate II, and working standard solutions, respectively, in mL.

(b) Peak areas are measured with a data system. Before calculating concentrations, review all chromatograms to make sure that *cis* and *trans* vitamin K_1 are baseline separated and that there are no interfering peaks. *Trans* vitamin K_1 concentrations cannot be calculated for any samples with interfering peaks or poor separation between the *cis* and *trans* isomers (*see* Figures **2015.09B–D**). Check the integration of the *cis* and *trans* vitamin K_1 peaks. *Cis* vitamin K_1 elutes 1 to 3 min before *trans* vitamin K_1 depending on the analytical column used. If the peak areas of the same standards injected before and after a set of samples have changed by more than 7%, the system was not equilibrated and the data are not acceptable.

(c) Calculation of *trans* vitamin K_1 standard concentration:

$$T = \frac{A_2}{(A_1 + A_2)}$$

where T is the *trans* vitamin K_1 fraction, A_1 is the *cis* vitamin K_1 peak area, and A_2 is *trans* vitamin K_1 peak area. The *trans* vitamin K_1 fraction is calculated for all standards, and the calculated *trans* vitamin K_1 fraction for all standards is averaged together (T_a) and used to calculate the *trans* vitamin K_1 concentration of all standards.

(d) *Trans* vitamin K_1 standard concentration:

$$C_{ST} = C_S \times T_a$$

where C_{ST} is the concentration of *trans* vitamin K_1 in the working standard C_S in µg/L and C_s is the working standard concentration in µg/L.

(e) *Preparation of standard curves*.—For each working standard concentration, average the peak areas from each two consecutive sets of standards. Prepare a standard curve by performing a linear least-squares regression on *trans* concentration versus averaged peak areas. A standard curve must have an r^2 of 0.999 or better to be acceptable.

(f) Calculation of *trans* vitamin K_1 in a sample:

$$C_p = \frac{C_c \times 10.0 \times R}{S \times P}$$

where C_p is the concentration of *trans* vitamin K_1 in µg/kg, C_c is the concentration of *trans* vitamin K_1 in the injected sample determined from the standard curve in µg/L, 10.0 is the dilution volume of the sample in mL, R is the final dilution weight of a product reconstitution in g (if necessary), S is the sample size in g, and P is the weight of product that is reconstituted in g (if necessary).

References: *J. AOAC Int*. **98**, 1382(2015)
DOI: 10.5740/jaoacint.15-130

AOAC SMPR 2014.001
J. AOAC Int. **98**, 1036 (2015)
DOI: 10.5740/jaoac.int.SMPR2014.001

Posted: October 12, 2015

INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Vitamin K_1 in Infant, Pediatric, and Adult Nutritionals by HPLC with Fluorescence Detection: Single-Laboratory Validation, First Action 2015.09

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This normal-phase HPLC method with postcolumn reduction and fluorescence detection allows for the quantitative determination of *trans* vitamin K_1 in infant, pediatric, and adult nutritionals. Vitamin K_1 is extracted from products with iso-octane after precipitation of proteins and release of lipids with methanol. Prepared samples are injected onto a silica HPLC column where *cis* and *trans* vitamin K_1 are separated with an iso-octane–isopropanol mobile phase. The column eluent is mixed with a dilute ethanolic solution of zinc chloride, sodium acetate, and acetic acid, and vitamin K_1 is reduced to a fluorescent derivative in a zinc reactor column. The resulting hydroquinone is then detected by fluorescence at an excitation wavelength of 245 nm and an emission wavelength of 440 nm. During a single-laboratory validation of this method, repeatability and intermediate precision ranged from 0.6 to 3.5% RSD and 1.1 to 6.0% RSD, respectively. Mean overspike recoveries ranged from 91.9 to 106%. The method demonstrated good linearity over a standard range of approximately 2–90 µg/L *trans* vitamin K_1 with r^2 averaging 0.99995 and average calibration errors of <1%. LOQ and LOD in ready-to-feed nutritionals were estimated to be 0.03 and 0.09 µg/100 g, respectively. The method met AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals *Standard Method Performance Requirements*® and was approved as a first action method at the 2015 AOAC Mid-Year Meeting.

Vitamin K_1 is an antihemorrhagic vitamin first isolated in 1939 after it was discovered that chicks fed diets previously extracted with nonpolar solvents developed subdural or muscular hemorrhages. Vitamin K_1, which is also known as phylloquinone and phytonadione, consists of a methyl-substituted naphthoquinone nucleus attached to a side chain of three saturated and one unsaturated isoprene units and is a yellow viscous oil. Although vitamin K_1 occurs naturally in the *trans* form, during synthesis of vitamin K_1 both the *cis* and *trans* isomers are formed with the *trans* isomer being the major product. Vitamin K_1 is insoluble in water and sparingly soluble in methanol and ethanol. It is soluble in vegetable oils and organic solvents such as pentane, hexane, iso-octane, and 2-propanol. Vitamin K_1 has five ultraviolet absorption maxima which are at 242, 248, 260, 269, and 325 nm and can be reduced to a fluorescent hydroquinone. Vitamin K_1 is stable to air, heat, oxidizing agents, and moisture, but its activity is destroyed by light (especially UV radiation), reducing agents, and alkalies (1).

Good sources of vitamin K_1 are alfalfa, cabbage, cauliflower, green vegetables, tomatoes, cheese, dairy products, meat, egg yolks, and canola and soy oil. Vitamin K_1 is also found in bacteria and is synthesized in the intestinal tract by microorganisms. *Trans* vitamin K_1 is biologically active, while the *cis* form has little if any activity (1).

At the September 2013 AOAC Annual Meeting, an AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) working group developed *Standard Method Performance Requirements* (SMPR®; 2) for *trans* vitamin K_1 and required separation of the *cis* and *trans* isomers since *cis* vitamin K_1 has little if any biological activity. SPIFAN approved AOAC SMPR 2014.001 at the March 2014 AOAC Mid-Year Meeting. Subsequently, AOAC issued a call for methods.

In response to AOAC's call for methods, a new vitamin K_1 method that combined the strengths of the two current AOAC Official Vitamin K_1 methods, **992.27** and **999.15**, was developed and validated. AOAC **992.27** uses liquid–liquid extraction in separatory funnels, open column cleanup, normal phase (NP) chromatography, and UV absorbance to extract, separate, and quantitate *trans* vitamin K_1 (3). Although the AOAC **992.27** sample preparation procedure provides better recovery of vitamin K_1 in more complex infant, pediatric, and adult nutritional matrixes than **999.15** and the sample preparation solvents are compatible with the NP chromatography, UV detection is not very specific and the sample preparation procedure is labor-intensive. AOAC **999.15** uses an enzyme digestion and liquid–liquid extraction in glass tubes, reversed-phase chromatography, and fluorescence detection after postcolumn reduction with zinc to extract, separate, and quantitate *trans* or total vitamin K_1 (4). Although AOAC **999.15** uses a more specific detection system and a simpler sample preparation procedure, it will not separate *cis* and *trans* vitamin K_1 if a C_{18} column is used; sample extracts must be dried down and the residue dissolved in a solvent compatible with

Received May 20, 2015. Accepted by SG July 8, 2015.
This method was approved by the Expert Review Panel for Infant Formula and Adult Nutritionals as First Action.
The Expert Review Panel for Infant Formula and Adult Nutritionals invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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DOI: 10.5740/jaoacint.15-130

RP chromatography, and it has been shown to under-recover vitamin K in some more complex infant, pediatric, and adult nutritional matrixes (5, 6).

This new NP HPLC method with postcolumn reduction and fluorescence detection allows for the quantitative determination of *trans* vitamin K_1 in infant, pediatric, and adult nutritionals. Vitamin K_1 is extracted from products with iso-octane after precipitation of proteins and release of lipids with methanol. Prepared samples are injected onto a silica HPLC column where *cis* and *trans* vitamin K_1 are separated with an iso-octane–isopropanol mobile phase. The column eluent is mixed with a dilute ethanolic solution of zinc chloride, sodium acetate, and acetic acid, and vitamin K_1 is reduced to a fluorescent derivative in a zinc reactor column. The resulting hydroquinone is then detected by fluorescence at an excitation wavelength of 245 nm and an emission wavelength of 440 nm.

Table 1. Method performance requirements: *trans*-vitamin K_1 [a]

Analytical range		1–100[b]
LOQ		≤1[b]
RSD_r, %	1–10[b]	≤8
	>10[b]	≤5
Recovery, %		90 to 110 of mean spiked recovery over the range of the assay
RSD_R, %	1–10[b]	≤15
	>10[b]	≤10

[a] Concentrations apply to (a) RTF liquids "as is", (b) reconstituted powders (25 g into 200 g of water), and (c) liquid concentrates diluted 1:1 by weight.

[b] µg/100 g reconstituted final product.

Single-Laboratory Validation (SLV)

Experimental

To verify the applicability of this method, an SLV with all 12 SPIFAN infant, pediatric, and adult matrixes was completed.

To establish method precision, all fortified and unfortified matrixes were prepared and analyzed in duplicate on 6 days. National Institute of Standards and Technology Standard Reference Material (SRM) 1849a (Infant/Adult Nutritional Formula) was reconstituted by dissolving the entire contents of the sachet (10 g) in 90 mL water. All other powders were reconstituted by dissolving 25 g powder in 200 mL laboratory water. New reconstitutions were prepared each day.

Method accuracy was established by spiking aliquots of each SPIFAN matrix with vitamin K_1 at approximately 50 or 100% of the previously determined *trans* vitamin K_1 level. Approximately 40–250 µL vitamin K_1, dissolved in ethanol, was added to 25 g aliquots of each SPIFAN matrix, and all sample and spike weights were recorded. On 2 days each sample matrix was spiked at 100% of the previously determined *trans* vitamin K_1 level, and on the third day each matrix was spiked at 50%. On each day spike blanks were also prepared by adding 75–250 µL vitamin K_1, dissolved in ethanol, to 25 mL iso-octane. Spiked samples and blanks were thoroughly mixed and stored refrigerated for at least 24 h to allow vitamin K_1 incorporation into the sample matrix. After at least 24 h, spiked and unspiked samples were prepared and analyzed in duplicate as described in the method. Spike blanks were diluted to appropriate concentrations with iso-octane and analyzed along with the spiked and unspiked sample preparations.

Method linearity was evaluated by injecting five or six standards with *trans* vitamin K_1 concentrations ranging from approximately 2 to 90 µg/L before and after every set of samples analyzed during validation. Calibration curves were

Table 2. *Trans* vitamin K_1 SLV data—precision

Sample type	No. of replicates (duplicates on multiple days)	Mean, µg/100 g RTF	SD_r	RSD_r, %	SD_{IP}	RSD_{IP}, %
Child formula powder, placebo	6	2.03	0.040	2.0	0.045	2.2
Infant elemental powder, placebo	6	2.03	0.020	1.0	0.025	1.2
Adult nutritional RTF high protein, placebo	6	3.47	0.021	0.6	0.040	1.2
Adult nutritional RTF high fat, placebo	6	3.07	0.034	1.1	0.034	1.1
Infant formula RTF milk based, placebo	6	2.16	0.038	1.8	0.038	1.8
SRM 1849a	12	1.11[a]	0.022	2.0	0.025	2.3
Adult nutritional powder milk protein based	12	3.26	0.097	3.0	0.142	4.4
Infant formula powder partially hydrolyzed milk based	12	7.69	0.123	1.6	0.170	2.2
Infant formula powder partially hydrolyzed soy based	12	8.99	0.141	1.6	0.226	2.5
Adult nutritional powder low fat	12	2.92	0.102	3.5	0.102	3.5
Child formula powder	12	2.66	0.049	1.8	0.050	1.9
Infant elemental powder	12	7.57	0.129	1.7	0.451	6.0
Infant formula powder milk based	12	6.09	0.105	1.7	0.131	2.2
Infant formula powder soy based	12	6.26	0.105	1.7	0.211	3.4
Infant formula RTF milk based	12	9.01	0.168	1.9	0.189	2.1
Adult nutritional RTF high protein	16	9.10	0.283	3.1	0.299	3.3
Adult nutritional RTF high fat	12	10.7	0.120	1.1	0.179	1.7

[a] Results reported as mg/kg powder.

Table 3. Trans vitamin K_1 SLV data–accuracy

			Spike level			
			100%		50%	
Sample type	No. of replicates (duplicates on multiple days)	Native level, µg/100 g RTF	Recovery, %	RSD, %	Recovery, %	RSD, %
Child formula powder	6	2.66	98.2	5.9	96.2	7.1
Infant elemental powder	6	7.57	93.2	7.6	94.0	2.6
SRM 1849a	6	1.11[a]	104	2.9	95.5	1.8
Adult nutritional powder milk protein based	6	3.26	96.4	2.0	95.1	1.8
Infant formula powder partially hydrolyzed milk based	6	7.69	96.6	3.6	91.9	3.0
Infant formula powder partially hydrolyzed soy based	6	8.99	97.9	1.0	96.1	2.6
Adult nutritional powder low fat	6	2.92	98.0	2.5	95.2	2.6
Infant formula powder milk based	6	6.09	97.6	1.0	102	2.3
Infant formula powder soy based	6	6.26	97.9	1.7	102	0.3
Infant formula RTF milk based	6	9.01	100	1.3	104	0.4
Adult nutritional RTF high protein	6	9.10	96.7	2.7	106	0.7
Adult nutritional RTF high fat	6	10.7	98.2	1.2	93.8	4.0

[a] Results reported as mg/kg powder.

constructed from these standards, and the regression parameters from least-squares fittings were used to back calculate the concentration of each working standard to determine calibration errors at each level. It should be noted that all commercially available vitamin K_1 standards contain a mixture of *cis* and *trans* vitamin K_1. The percentage of *trans* vitamin K_1 in the standard was determined experimentally for each run using *cis* and *trans* peak areas from all working standard chromatograms. The experimentally determined ratio of *trans* vitamin K_1 was then used to calculate the *trans* vitamin K_1 standard concentrations of the working standards.

Trans vitamin K_1 LOD and LOQ were determined experimentally by injecting a very low level vitamin K_1 standard of known concentration and measuring the S/N. *Trans* vitamin K_1 LOD and LOQ in the standard solution were calculated by multiplying the background noise by 3 (LOD) or 10 (LOQ) and dividing by the sensitivity, which was defined as the ratio of the analytical signal to the concentration of the analyte producing the signal. Product LOD and LOQ were extrapolated from the standard LOD and LOQ using a typical sample weight and dilution volume.

Ruggedness (or robustness) was not explicitly studied; however, several parameters relevant to this were varied during the SLV in order to factor as much uncertainty as possible into the method performance metrics. Samples were prepared by two analysts and analyzed with silica columns from three different vendors. New mobile phase and postcolumn reagents were made daily, and two sets of stock, intermediate, and working standards were prepared and used during validation.

All of the unfortified matrixes were expected to contain some *trans* vitamin K_1 and could not be used to unambiguously establish method specificity; however, this method uses a very specific detection technique. Relatively few compounds

Table 4. Summary of *trans* vitamin K_1 relative (%) calibration errors by level (30 curves)[a]

Calibration level[b]	Mean	Median	Minimum	Maximum	*P* [c]
1	0.247	1.55	–7.94	5.04	0.698
2	0.484	0.879	–3.65	2.93	0.0930
3	0.117	0.0280	–1.00	2.21	0.350
4	0.0972	–0.0710	–1.08	1.9	0.443
5	–0.328	–0.271	–1.70	1.47	0.0460
6	0.0561	0.101	–0.421	0.372	0.201
Run average	0.104	0.364	–1.73	1.18	0.428

[a] r^2 for the 30 curves ranged from 0.99985 to 1.00000, with an average of 0.99994.

[b] Levels 1–6 corresponds to *trans* vitamin K_1 concentrations of 2–3, 6–8, 11–13, 22–30, 37–45, and 74–88 µg/L.

[c] *P* value for one sample *t*-test relative to zero.

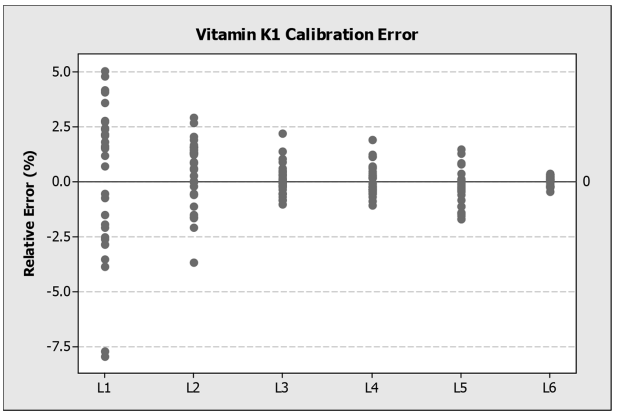

Figure 1. Vitamin K_1 standard curve calibration errors; L = level.

naturally fluoresce or form fluorescent derivatives when exposed to zinc.

Acceptance Criteria

Previously established method performance requirements for vitamin K (SMPR 2014.001) are summarized in Table 1.

Results

The SRM repeatability and recovery requirements were met for all 12 matrixes. Repeatability data are summarized in Table 2. Repeatability precision (RSD_r) and intermediate precision (RSD_{IP}) ranged from 0.6 to 3.5% and 1.1 to 6.0%, respectively. Pooled across all samples, the RSD_r was 2.0% and the RSD_{IP} was 2.8%. Mean spike recovery data are summarized in Table 3 and ranged from 91.9 to 106%, averaging 97.8% across all samples. The method demonstrated good linearity over a standard range of approximately 2–90 µg/L *trans* vitamin K_1 with r^2 averaging 0.99995. These data are summarized in Table 4. Average calibration errors were <1% and are illustrated graphically in Figure 1.

The LOQ requirement was met. LOD and LOQ were estimated to be 0.1 and 0.4 µg/L, respectively, with standards and 0.03 and 0.09 µg/100 g ready-to-feed (RTF) liquids, respectively, for products assuming a 4 g sample diluted to 10 mL.

AOAC Official Method 2015.09
Trans Vitamin K_1
in Infant, Pediatric, and Adult Nutritionals
HPLC with Fluorescence Detection
First Action 2015

(Applicable to the determination of *trans* vitamin K_1 in infant, pediatric, and adult nutritional formulas.)

Caution: Refer to Material Safely Data Sheets (MSDS) of chemicals prior to use and use the suggested personal protective equipment. Zinc powder should be handled in a fume hood; it is self-heating and may catch fire. Zinc powder should not be allowed to come into contact with water, which may release flammable gases that may spontaneously ignite.

See Tables 2 and 3 for infant, pediatric, and nutritional matrixes for which the method has been validated.

A. Principle

This normal-phase (NP) HPLC method with postcolumn reduction and fluorescence detection allows for the quantitative determination of *trans* vitamin K_1 in infant, pediatric, and adult nutritionals. Vitamin K_1 is extracted from products with iso-octane after precipitation of proteins and release of lipids with methanol. Prepared samples are injected onto a silica HPLC column where *cis* and *trans* vitamin K_1 are separated with an iso-octane–isopropanol mobile phase. The column eluent is mixed with a dilute ethanolic solution of zinc chloride, sodium acetate, and acetic acid, and vitamin K_1 is reduced to a fluorescent derivative in a zinc reactor column. The resulting fluorescent compound is then detected by fluorescence at an excitation wavelength of 245 nm and an emission wavelength of 440 nm.

B. Apparatus and Materials

(a) *HPLC system*.—Two isocratic pumps; autosampler capable of injecting 20 µL; fluorescence detector; high-pressure mixing tee; and postcolumn reactor column 20 × 4 mm stainless steel (Waters, Milford, MA; Part No. WAT084550 or equivalent). The system should be configured as shown in Figure **2015.09A**.

(b) *Analytical column*.—Silica 150 × 3.0 mm, 3 µm, 60 Å, or equivalent.

(c) *Analytical balance*.—Capable of weighing to the nearest 0.00001 g.

(d) *Beakers*.—Glass, assorted sizes.

(e) *Centrifuge*.

(f) *Centrifuge tubes and caps*.—50 mL glass tubes with Teflon-lined caps.

(g) *Cylinders*.—Graduated, glass, assorted sizes.

(h) *Gas regulator*.—Helium.

(i) *Helium sparge*.—Tubing and filtering assembly.

(j) *Magnetic stirrer*.—Variomag Telesystem (Daytona Beach, FL) HP 60 S (Part No. HP 46040U) with tube rack (Part No. HP 86029) and egg-shaped Teflon stirring bars (Part No. PI20110) or equivalent.

(k) *Pipet*.—Disposable glass, Pasteur.

(l) *Pipet*.—Mechanical, variable volume, 0.5–5 mL and 10–100 µL.

(m) *Pipet*.—Repeating 5 and 25 mL or equivalent.

(n) *Volumetric flasks*.—Glass, Class A, assorted sizes.

(o) *Volumetric pipets*.—Glass, Class A, assorted sizes.

(p) *Vortex mixers*.

(q) *Yellow lights or yellow shields with cutoff of at least 440 nm*.

C. Reagents

(a) *Acetic acid*.—Glacial, ACS.

(b) *Helium*.—Zero grade or equivalent.

(c) *Iso-octane (2,2,4-trimethylpentane)*.—HPLC grade.

(d) *Isopropanol (isopropyl alcohol)*.—HPLC grade.

(e) *Methanol*.—HPLC grade.

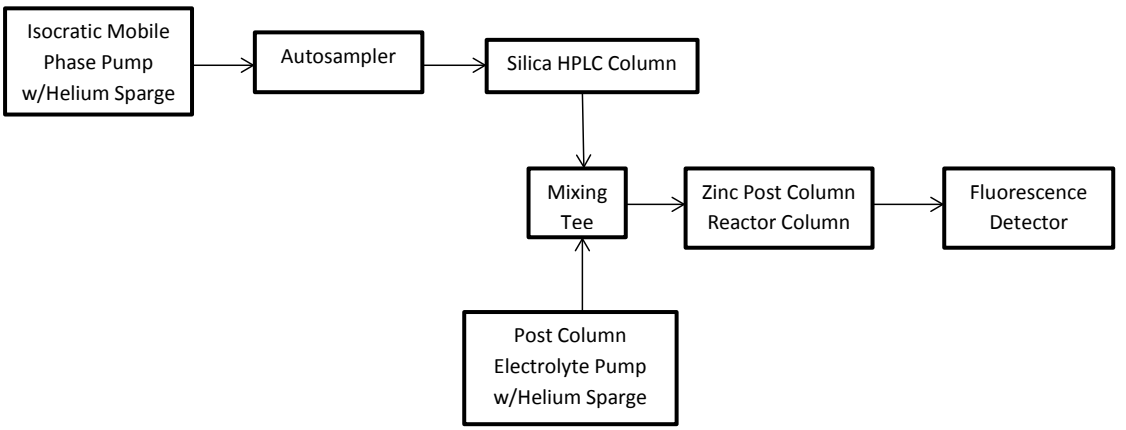

Figure 2015.09A. Vitamin K system configuration.

(f) *Phytonadione/phylloquinone (vitamin K_1)*.—U.S. Pharmacopeia Reference, Official Lot. Store per label instructions.

(g) *Laboratory water*.

(h) *Reagent alcohol*.—ACS.

(i) *Sodium acetate anhydrous*.—ACS.

(j) *Zinc*.—Sigma-Aldrich (St. Louis, MO) Part No. 324930, <150 µm, 99.995% or equivalent.

(k) *Zinc chloride*.—ACS.

D. Standard and Solution Preparation

Note: Since vitamin K_1 is light-sensitive, all standards must be prepared, handled, and stored in the dark or under yellow shielded lighting (*see* **B**) unless otherwise stated. If the standards must be transported through or into an area without yellow shielded lighting, they must be wrapped tightly in foil. All standard solutions must be prepared using Class A volumetric glassware.

(a) *Mobile phase*.—Add about 900 mL iso-octane to a 1000 mL volumetric flask. Add 0.3–0.4 mL isopropanol to the volumetric flask and dilute to volume with iso-octane. (*Note*: The isopropanol concentration in the mobile phase can be adjusted slightly until baseline resolution of *cis* and *trans* vitamin K_1 from other peaks present in some samples is achieved. *See* Figures **2015.09B–D**).

(b) *Postcolumn electrolyte solution*.—Transfer 0.25 (±0.02) g zinc chloride and 0.10 (±0.05) g sodium acetate anhydrous to a 1000 mL volumetric flask with reagent alcohol. Add 75 (±5) µL concentrated acetic acid and dilute to volume with reagent alcohol. Add a stir bar and stir solution for about 30 min or until solution is clear and all salts are dissolved.

(c) *Vitamin K_1 (phytonadione) stock standard solution*.—Accurately weigh to 0.00001 g about 0.05500 g vitamin K_1 (phytonadione) into a 250 mL volumetric flask. Dissolve oil and dilute to volume with iso-octane. Store refrigerated in a tightly stoppered container protected from light. Expiration 6 months.

(d) *Vitamin K_1 (phytonadione) intermediate I standard solution*.—Dilute 1.0 mL vitamin K_1 stock standard to 100 mL with iso-octane. Prepare fresh each time new working standards are made.

(e) *Vitamin K_1 (phytonadione) intermediate II standard solution*.—Dilute 10.0 mL vitamin K_1 intermediate I standard to 50 mL with iso-octane. Prepare fresh each time new working standards are made.

(f) *Vitamin K_1 (phytonadione) working standard solutions*.—Dilute 1.0, 3.0, 6.0, 10.0, and 20.0 mL intermediate II standard to 100 mL with iso-octane. Store refrigerated in tightly stoppered containers protected from light. Expiration 3 months.

(*Note*: Transfer working standards to autosampler vials with Pasteur pipets or equivalent glass. Do not pour the standards from the volumetric flasks into vials.)

E. Procedure

[(*Note*: Because vitamin K_1 is light-sensitive, all samples must be prepared, handled, and stored in the dark or under yellow-shielded lighting (**B**) unless otherwise stated. If the samples must be transported through or into an area without yellow-shielded lighting, they must be wrapped tightly in foil.)]

(a) *Sample preparation*.—(*1*) Accurately weigh to 0.0001 g, up to 0.5 g homogeneous powder or up to 4 g of ready-to-feed (RTF) liquids or nonhomogeneous powders diluted to RTF concentrations into 50 mL centrifuge tubes. To powders weighed directly into the 50 mL centrifuge tubes, add 4 mL water and mix well. To liquids with sample weights that are less than 4 g, add enough water to the tubes so that the sample weight plus the amount of added water equals about 4 and mix well.

(*2*) Add 25 (±2.0) mL methanol to each sample just prior to vortexing or stirring. Methanol should not be added to more than two samples consecutively without vortexing or stirring. Cap each centrifuge tube. Vortex each sample at high speed for at least 30 s, and allow samples to sit undisturbed for at least 10 min, but no more than 40 min, after vortexing with methanol, or add a magnetic stir bar to each sample, place each capped sample onto a magnetic stir plate, and stir each sample for at least 10 min, but not more than 40 min, at a spin rate that causes a vortex.

(*3*) Add 10 (±0.05) mL iso-octane to each sample with a volumetric pipet and cap tubes. Iso-octane can be added to all samples before vortexing or stirring any of the samples. Vortex each sample for at least 45 s or stir each sample for at least 45 s at a spin rate that causes a vortex to form within the sample.

(*4*) Add 5 (±1) mL laboratory water to each sample and cap tubes. Laboratory water can be added to all the samples prior to vortexing or stirring. Vortex or shake each sample for at least

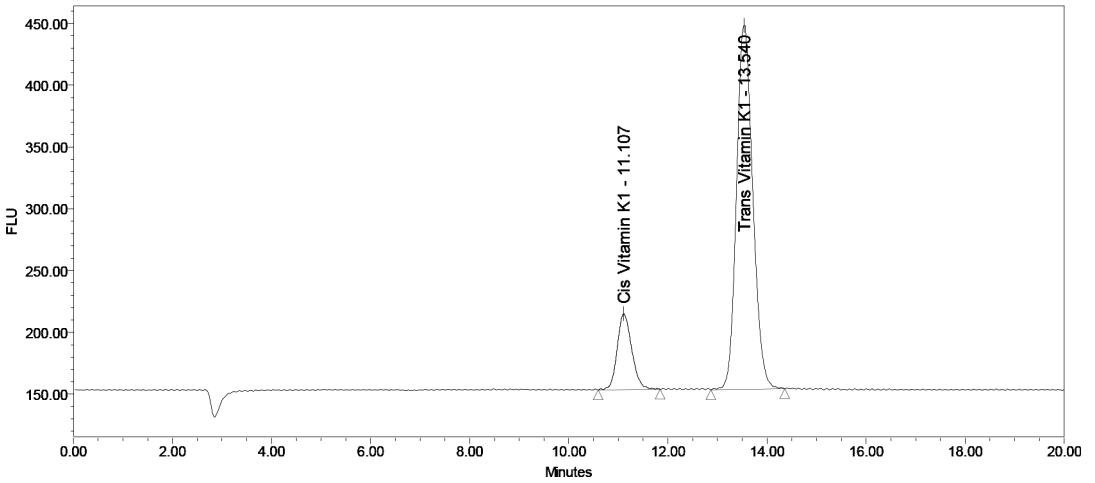

Figure 2015.09B. Chromatogram of vitamin K_1 standard.

20 s or stir each sample for at least 20 s at a spin rate that causes a vortex to form within the sample.

(*5*) Centrifuge the samples until a clean separation of the iso-octane and laboratory water–methanol layers results. The iso-octane layer should be a clear layer at the top of the centrifuge tube, and the laboratory water–methanol layer should be a cloudy layer below the iso-octane layer. (A good separation of solvent layers can usually be achieved by centrifuging samples for approximately 10 min at 800 relative centrifugal force.)

(*6*) Remove samples from the centrifuge and inspect the samples to verify that the iso-octane and laboratory water–methanol layers are separated. With a glass pipet, carefully rinse down the upper walls of the centrifuge tube with a portion of the iso-octane layer. If the layers become mixed together, centrifuge the sample again. Pipette a portion of the clear iso-octane layer into a labeled autosampler vial and cap the vial.

(b) *HPLC analysis*.

(*1*) *Instrumental operating conditions*.—(*a*) HPLC analytical column pump flow rate, 0.4 mL/min. (*b*) Postcolumn flow rate, 0.4 mL/min. (*c*) Injection volume, 20 µL. (*d*) Run time, 20 min. (*e*) Fluorescence excitation and emission, 245 and 440 nm, respectively.

(*2*) *Instrument startup*.—The system should be configured as shown in Figure **2015.09B**.

If necessary, remove used zinc and repack the postcolumn reactor column with fresh zinc. The zinc reactor column should be repacked whenever the S/N in the lowest standard is too high to accurately integrate the vitamin K_1 peak, when peak responses from injections of the same standard drop by more than 7% and the drop cannot be attributed to other system components, or when the system back pressure through the zinc reactor increases significantly and vitamin K_1 peak widths begin to increase. To repack the zinc reactor column, remove the hex nuts and retainers from both ends of the column and force the used zinc out of the column with a thin wire or similar apparatus. Flush the zinc reactor column with ethanol to remove residual zinc. Replace the hex nut and retainer on one end of the zinc reactor column. Carefully transfer a small amount of zinc powder to the reactor column with a spatula, and press down on the zinc in the column with an old HPLC piston or similar apparatus to pack it tightly. Continue adding zinc and pressing it down until the level of zinc is even with the top of the column. After the reactor column is full, replace the second retainer and hex nut. The more tightly zinc is packed into the reactor column, the more symmetrical the vitamin K_1 peaks

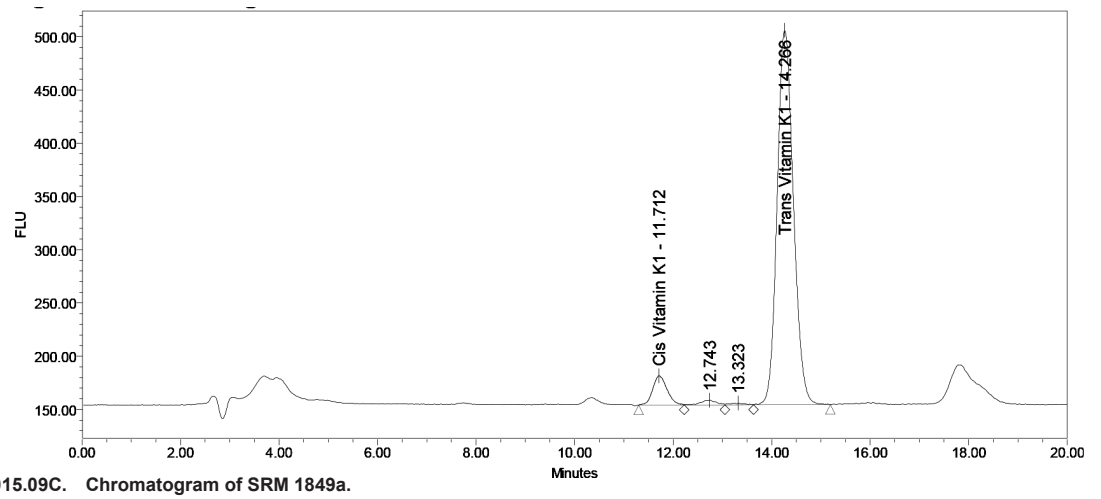

Figure 2015.09C. Chromatogram of SRM 1849a.

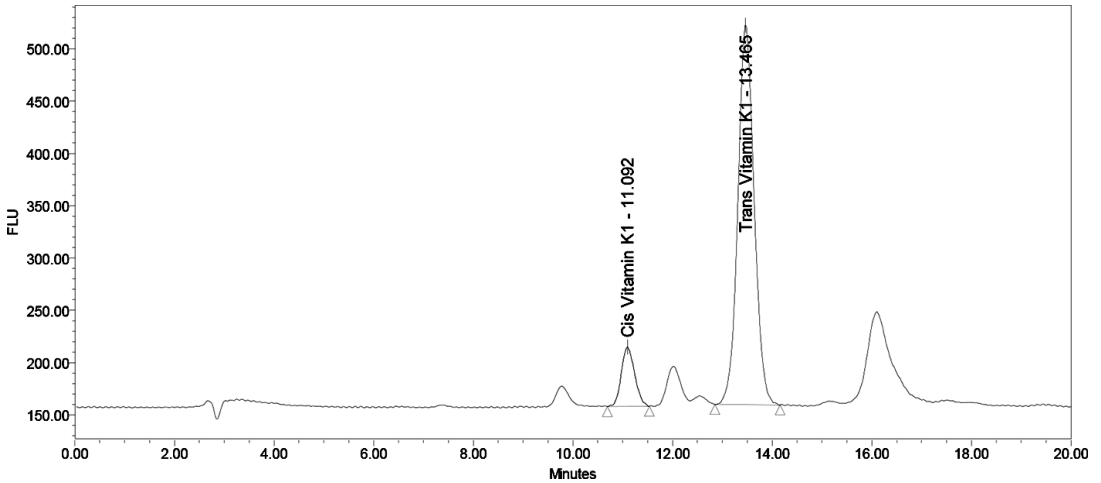

Figure 2015.09D. Chromatogram of an adult nutritional.

will be. Degas the mobile phase and postcolumn electrolyte solutions by bubbling helium through them at a flow rate just fast enough to cause small ripples on the surface of the mobile phase and postcolumn solutions. To maximize the life of the zinc reactor column, degas the mobile phase and postcolumn electrolyte solution for at least 30 min before connecting the zinc reactor column or do not pump mobile phase and postcolumn electrolyte solutions until at least 30 min after degassing begins. Once the mobile phase and postcolumn electrolyte solutions have been degassed, allow the column and postcolumn reactor to equilibrate with mobile phase flowing at 0.4 mL/min and postcolumn electrolyte solution flowing at 0.4 mL/min for at least 30 min prior to the first injection if the zinc reactor has been used for previous analyses or several hours if the zinc postcolumn reactor has been freshly packed. Once the mobile phase and postcolumn solutions have been degassed, reduce the helium flow rate so that only a small stream of helium bubbles are visible in the mobile phase and postcolumn solutions and there is minimal disturbance to the surface of these solutions. Bubble helium very slowly through the mobile phase and postcolumn electrolyte solutions continuously throughout the entire run. Once the run has started, do not adjust the helium flow rate. Allow the fluorescence detector lamp to warm up 30 min prior to the first injection. (*Note*: When the mobile phase and postcolumn electrolyte solution are continuously sparged with helium throughout a run, it is not necessary to pack the postcolumn reactor with zinc at the beginning of every run. It should be possible to analyze hundreds of samples before the zinc reactor column must be repacked.)

(*3*) *HPLC of standards and samples*.—Inject the most concentrated standard (approximately 80 µg/L) onto the column and observe the response on the fluorescence detector. If necessary, adjust the detector gain and sensitivity settings so that the standard response is within the range of the detector. Once the detector settings have been determined, inject the most concentrated standard 3–4 times and note the peak areas. If the system is equilibrated, the RSD of the standard peak areas should be ≤2%, and the peak areas should not steadily increase or decrease by more than 4% from the first injection to the third or fourth injection. If the RSD is >2%, locate the source of the imprecision and correct it before beginning the sample analysis. If peak areas steadily increase or decrease by more than 4%, the system is not equilibrated and must be allowed to equilibrate longer. Once the system has reached equilibrium and the RSD is ≤2%, inject a set of standards, unknown samples, and another set of standards. Every set of unknown samples must be bracketed by standards.

(*4*) *Instrument shut down*.—After analyzing a set of samples, simultaneously turn off the flow on the mobile phase and postcolumn electrolyte solution pumps. Remove the helium sparge lines from the mobile phase and postcolumn electrolyte solution and turn off the helium. Turn off the fluorescence detector lamp.

F. Calculations

The vitamin K_1 concentrations of samples analyzed on the HPLC system are determined by comparison of peak areas from samples of known weight with the peak areas of standards of known concentration. Because the *cis* and *trans* vitamin K_1 retention times may shift slightly during a run, peak areas must be used to quantitate *trans* vitamin K_1.

(a) Calculation of the standard concentrations:

$$C_s = \frac{W \times (V_1 \times V_2 \times V_3) \times 1{,}000{,}000{,}000}{(D_1 \times D_2 \times D_3 \times D_4)}$$

where C_s is the working standard concentration in µg/L; W is the weight of standard in g; V_1, V_2, and V_3 are the aliquots of stock, intermediate I, and intermediate standard II solutions, respectively, in mL; 1,000,000,000 is the conversion factor from g/mL to mcg/L; and D_1, D_2, D_3, and D_4 are the dilution volumes of the stock, intermediate I, intermediate II, and working standard solutions, respectively, in mL.

(b) Peak areas are measured with a data system. Before calculating concentrations, review all chromatograms to make sure that *cis* and *trans* vitamin K_1 are baseline separated and that there are no interfering peaks. *Trans* vitamin K_1 concentrations cannot be calculated for any samples with interfering peaks or poor separation between the *cis* and *trans* isomers (*see* Figures **2015.09B–D**). Check the integration of the *cis* and *trans* vitamin K_1 peaks. *Cis* vitamin K_1 elutes 1 to 3 min before *trans* vitamin K_1 depending on the analytical column used. If the peak

areas of the same standards injected before and after a set of samples have changed by more than 7%, the system was not equilibrated and the data are not acceptable.

(c) Calculation of *trans* vitamin K_1 standard concentration:

$$T = \frac{A_2}{(A_1 + A_2)}$$

where T is the *trans* vitamin K_1 fraction, A_1 is the *cis* vitamin K_1 peak area, and A_2 is *trans* vitamin K_1 peak area. The *trans* vitamin K_1 fraction is calculated for all standards, and the calculated *trans* vitamin K_1 fraction for all standards is averaged together (T_a) and used to calculate the *trans* vitamin K_1 concentration of all standards.

(d) *Trans* vitamin K_1 standard concentration:

$$C_{ST} = C_S \times T_a$$

where C_{ST} is the concentration of *trans* vitamin K_1 in the working standard C_S in µg/L and C_s is the working standard concentration in µg/L.

(e) *Preparation of standard curves*.—For each working standard concentration, average the peak areas from each two consecutive sets of standards. Prepare a standard curve by performing a linear least-squares regression on *trans* concentration versus averaged peak areas. A standard curve must have an r^2 of 0.999 or better to be acceptable.

(f) Calculation of *trans* vitamin K_1 in a sample:

$$C_p = \frac{C_c \times 10.0 \times R}{S \times P}$$

where C_p is the concentration of *trans* vitamin K_1 in µg/kg, C_c is the concentration of *trans* vitamin K_1 in the injected sample determined from the standard curve in µg/L, 10.0 is the dilution volume of the sample in mL, R is the final dilution weight of a product reconstitution in g (if necessary), S is the sample size in g, and P is the weight of product that is reconstituted in g (if necessary).

G. Validation Data

See Tables 2 and 3.

Conclusions

AOAC **2015.09** met all of the SPIFAN SMPR and was approved for First Action status by an AOAC expert review panel at the AOAC Mid-Year Meeting in March 2015.

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(5) Schimpf, K.J., Thompson, L.B., & Schmitz, D.J. (2010) *J. AOAC Int*. **93**, 650–662

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AOAC Official Method 2015.10
Free and Total Choline and Carnitine
in Infant Formula and Adult/Pediatric Nutritional Formula
Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)
First Action 2015

A. Apparatus

(a) *LC system.* – Prominence (Shimadzu, Kyoto, Japan) or equivalent.

(b) *MS/MS system*. – API 4000 with Electrospray Ionization (ESI) (ABSciex, Framingham, MA) or equivalent.

(c) *Mass Spectrometry Software.* – Analyst (ABSciex) or equivalent.

(d) *Analytical column*. – Zorbax 300-SCX, 3.0 x 50 mm, 5 µm (Agilent, Santa Clara, CA) or equivalent.

(e) *Microwave*. – MARS6 (CEM, Mathews, NC) or equivalent.

(f) *Microwave turntable, liner, and cap*. – MARSXpress, 55 mL PFA Teflon®, 40 position (CEM or equivalent).

(g) *Vortex mixer.* – (VWR, West Chester, PA) or equivalent.

(h) *Analytical balances.* – Model CPA225D (Sartorius, Goettingen, Germany) or equivalent.

(i) *Horizontal shaker.* – Model 6010 (Eberbach, Ann Arbor, MI) or equivalent.

(j) *Magnetic stir plate.* – Model PC-420D (Corning, Corning, NY) or equivalent.

(k) *Positive displacement pipets.* – Microman, various sizes (Gilson, Middleton, WI) or equivalent.

(l) *Repeater positive displacement pipet.* – Repeater Plus (Eppendorf, Hamburg, Germany) or equivalent.

(m) *Polypropylene tubes.* – Digitube, assorted sizes (SCP Science, Montreal, Canada) or equivalent.

(n) *Mobile phase containers*. – 2 L glass (VWR) or equivalent.

(o) *Syringe filters*. – 0.45 µm PTFE and GHP (Pall, Plano, TX) or equivalent.

(p) *Disposable syringes*. – 3 mL (BD Biosciences, Franklin Lakes, NJ) or equivalent.

(q) *Graduated cylinders*. – Assorted sizes (VWR) or equivalent.

(r) *Magnetic stir bars*. – 7.9 x 50 mm (VWR) or equivalent.

(s) *Autosampler vials/caps.* – 1.5 mL silanized crimp top (VWR) or equivalent.

(t) *Microcentrifuge tubes*. – 1.5 mL polypropylene (VWR) or equivalent.

(u) *Bottle top dispenser*. – 5 mL acid resistant (Brand, Essex, CT) or equivalent.

(v) *Desiccator*. – glass (VWR) or equivalent.

Note: Nonspecific binding can occur with these analytes when using glassware, so plasticware should be used at all times for standard/sample preparation. All laboratory plasticware should be single-use whenever possible. Positive displacement pipets are also mandatory for pipeting to avoid contamination and for accuracy with organic solvents.

B. Chemicals and Reagents

(a) *Water*. – Optima MS grade (Thermo Fisher Scientific, Waltham, MA) or equivalent.

(b) *Acetonitrile*. – Optima MS grade (Thermo Fisher Scientific) or equivalent.

(c) *Ammonium formate*. – Optima MS grade (Thermo Fisher Scientific) or equivalent.

(d) *Formic acid*. – Optima MS grade (Thermo Fisher Scientific) or equivalent.

(e) *Nitric acid*. – 70% w/w, ACS grade (Avantor, Center Valley, PA) or equivalent.

(f) *Isopropanol*. – Optima MS grade (Thermo Fisher Scientific) or equivalent.

(g) *Desiccant*. – (VWR) or equivalent.

(h) *Reference Standard*. – L-Carnitine (USP, Rockville, MD) or equivalent.

(i) *Reference Standard*. – Choline Bitartrate (TCI, Tokyo, Japan) or equivalent.

(j) *Reference Internal Standard*. – L-Carnitine-d3 HCl (CDN Isotopes, Pointe Claire, Québec, Canada) or equivalent.

(k) *Reference Internal Standard*. – Choline-1,1,2,2-d4 Chloride (CDN Isotopes) or equivalent.

Note: All use of water in this method must be high purity MS grade water. It is recommended that all preparation steps with nitric acid be performed within a fume hood, and the necessary personal protective equipment used when handling.

C. Mobile Phase Preparation

Mobile phase A (5 mM ammonium formate in 50:50 (v/v) water:acetonitrile with 0.2% formic acid) was prepared by weighing 0.63 g of ammonium formate into a 1 L graduated cylinder. Water was added along with a stir bar and mixed to dissolve before diluting to volume with water. The solution was transferred to a 2 L mobile phase container along with 1 L of acetonitrile, 4 mL of formic acid, stir bar, and then thoroughly mixed. Mobile phase B (30 mM ammonium formate in 50:50 (v/v) water:acetonitrile with 0.2% formic acid) was prepared by weighing 3.78 g of ammonium formate into a 1 L graduated cylinder. Water was added along with a stir bar and mixed to dissolve before diluting to volume with water. The solution was transferred to a 2 L mobile phase container along with 1 L of acetonitrile, 4 mL of formic acid, stir bar, and then thoroughly mixed. Mobile phase B was also used for the rinse solutions in the autosampler.

D. Preparation of Standard Solutions

The carnitine stock standard was prepared at a concentration of 25 mg/mL by weighing 0.25 g of L-carnitine into a 20 mL polypropylene tube followed by 10 mL of water to dissolve. The purity of L-carnitine from the Certificate of Analysis (CoA) and moisture determined by Karl Fisher immediately at

the time of weighing was used to calculate the final concentration of carnitine. The choline stock standard was prepared at a concentration of 25 mg/mL choline by weighing 0.62 g of choline bitartrate into a 20 mL polypropylene tube followed by 10 mL of water to dissolve. The purity of choline bitartrate from the CoA along with a molecular weight conversion from choline bitartrate to choline of 0.41133 was used to calculate the final concentration of choline. Intermediate working standards were prepared at concentrations of 10, 20, 500, 2000, 4000, and 5000 µg/mL for each analyte using both the stock and higher concentration intermediate working standard solutions using appropriate volumes into 20 mL polypropylene tubes with water as the diluent. All stock and intermediate standard solutions were stable for 2 months when stored at 5 ± 3°C and protected from light. Aliquots of the intermediate working standards were treated through the sample analysis, so the concentrations used for the calibration curves for both free and total analyses were the same numerical values as the intermediate working standards but in ng/mL. Internal stock standards were prepared at a concentration of 2 mg/mL by weighing 25 mg of L-carnitine-d3 and 35 mg choline-1,1,2,2-d4 into separate 20 mL polypropylene tubes. A volume of 10 mL of water was added to each to dissolve, and then both solutions quantitatively transferred to a 100 mL polypropylene tube and diluted to volume with water to prepare an intermediate solution at 200 µg/mL. The purity from the CoA was used to calculate the final concentration of each internal standard. Stability of these solutions was monitored while being stored at 5 ± 3°C and protected from light.

E. Sample Preparation

Powder infant formula (IF) and adult nutritionals (AN) were reconstituted by weighing 25 g and diluting with water to a final weight of 225 g. Viscous ready to feed (RTF) products that were being analyzed for total choline and carnitine were pre-diluted by weighing 1.0 g and diluting with water to a final weight of 5.0 g.

(a) *Free choline and carnitine*. – Samples were prepared by weighing 1.0 g of reconstituted product into a 50 mL polypropylene tube. Six additional tubes were designated for the working standards along with two tubes for the reagent blank and reagent blank + internal standard to monitor any interference or carry over. The working standards, reagent blank, and reagent blank + internal standard were included with each free analysis and treated the same as samples through the sample preparation. The working standard tubes received 50 µl of the appropriate intermediate working standard level. All tubes except the reagent blank received 50 µl of the intermediate internal standard solution. The tubes were diluted to 25 mL with water and thoroughly mixed on a horizontal shaker. The reagent blank + internal standard solution was used as the diluent if dilutions were needed. A 0.5 mL aliquot of the sample solution was mixed with 0.5 mL of acetonitrile in a microcentrifuge tube, and then filtered through a 0.45 µm GHP syringe filter into a silanized injection vial. Aliquots of 0.5 mL of the working standard and reagent blank solutions were mixed with 0.5 mL of acetonitrile directly in the silanized injection vials.

(b) *Total choline and carnitine*. – Samples were prepared by weighing 1.0 g of reconstituted or diluted product into a 55 mL MarsExpress liner. Six additional liners were designated for the working standards along with two liners for the reagent blank and reagent blank + internal standard to monitor

any interference or carryover. The working standards, reagent blank, and reagent blank + internal standard were included with each total analysis and treated the same as samples through the sample preparation. Liners designated for the working standards received 50 µl of the appropriate intermediate working standard level. All liners except the reagent blank received 50 µl of the intermediate internal standard solution. A 5 mL volume of water followed by 2.5 mL of 70% (w/w) nitric acid delivered with a bottle top dispenser were then added to each liner, capped, and vortexed to mix. The microwave program used was a ramp to temperature of 120°C over 10 minutes, followed by a 40 minute hold at a power of 1000 W, ending in a cool down (6). The contents of the vessels were transferred into 50 mL polypropylene tubes with water and diluted to a volume of 25 mL with water. A 0.5 mL aliquot of the sample solution was mixed with 0.5 mL of acetonitrile in a microcentrifuge tube, and then filtered through a 0.45 µm PTFE syringe filter into a silanized injection vial. Aliquots of 0.5 mL of the working standard and reagent blank solutions were mixed with 0.5 mL of acetonitrile directly in the silanized injection vials.

F. LC/MS/MS Parameters

A Shimadzu Prominence liquid chromatography system equipped with an Agilent Zorbax 300-SCX column (3.0 x 50 mm, 5 µm) was utilized. A flow rate of 1.0 mL/min was maintained over the 4.2 minute total run time. The mobile phase conditions were 100% mobile phase A until 1.0 minute, ramped to 100% mobile phase B by 1.5 minutes, and ramped back to 100% A by 3.0 minutes. A column temperature of 40°C, and an autosampler temperature of 5°C was maintained. A 1 µl injection was used. Autosampler rinse settings were adjusted to eliminate carryover as much as possible. An ABSciex API 4000 mass spectrometer with positive ion electrospray (ESI) ionization was used in multiple reaction monitoring (MRM) mode. The MS/MS overall settings used are described in Table **2015.10A**. The MS/MS settings may need to be modified except for ionization, mode, and gas types to obtain optimum chromatography and sensitivity. Figures **2015.10A** and **2015.10B** show typical extracted ion chromatograms (XIC) of NIST SRM 1849a for choline and carnitine.

G. Quantification and Confirmation

The quantification of choline and carnitine was accomplished by generation of calibration curves using the peak area ratio of the chosen transition (Table **2015.10B**) versus the corresponding deuterated internal standards. Least square regression analysis using a linear model with $1/x^2$ weighting was used for both analytes. Confirmation was achieved through analysis of ion ratios between samples and reference standards for at least one additional transition listed in the table. The concentration of each analyte in a sample was calculated by the following equation:

$$R = \frac{\mathrm{C} \times \mathrm{V} \times \mathrm{D}}{S} \times \frac{100}{10^6\ \mathrm{ng/mg}}$$

where R = results expressed in mg/100g, C = concentration of the analyte in the injected solution in ng/mL, V = volume of the initial extract in mL, S = sample weight in g, and D = dilution factor, the inverse of any dilution made. All results were calculated on a ready to feed or reconstituted basis of 25 g diluted to 225 g with water, except for SRM 1849a that was calculated back to powder basis.

References

J. AOAC Int. (future issue)

AOAC SMPR 2012.010
J. AOAC Int. **96**, 488(2013)
DOI: 10.5740/jaoac.int.SMPR2012.010

AOAC SMPR 2012.013
J.AOAC Int. **96**, 492(2013)
DOI: 10.5740/jaoac.int.SMPR2012.013

Posted: October 1, 2015

Table 2015.10A. MS/MS Settings

Ionization	Positive Ion Electrospray (ESI+)
Mode	MRM
IonSpray Voltage	1000 V
TurboIon Spray Temp	550°C
Declustering Potential	120 V
Dwell Time	100 msec
Entrance Potential	10 V
Collision Cell Exit Potential	25 V
Collision Gas	Nitrogen, 5 psig
Curtain Gas	Nitrogen, 20 psig
Nebulizing Gas (Gas 1)	Nitrogen, 60 psig
Auxiliary Gas (Gas 2)	Nitrogen, 60 psig
Needle Position	Y = 5 mm, X = 5 mm

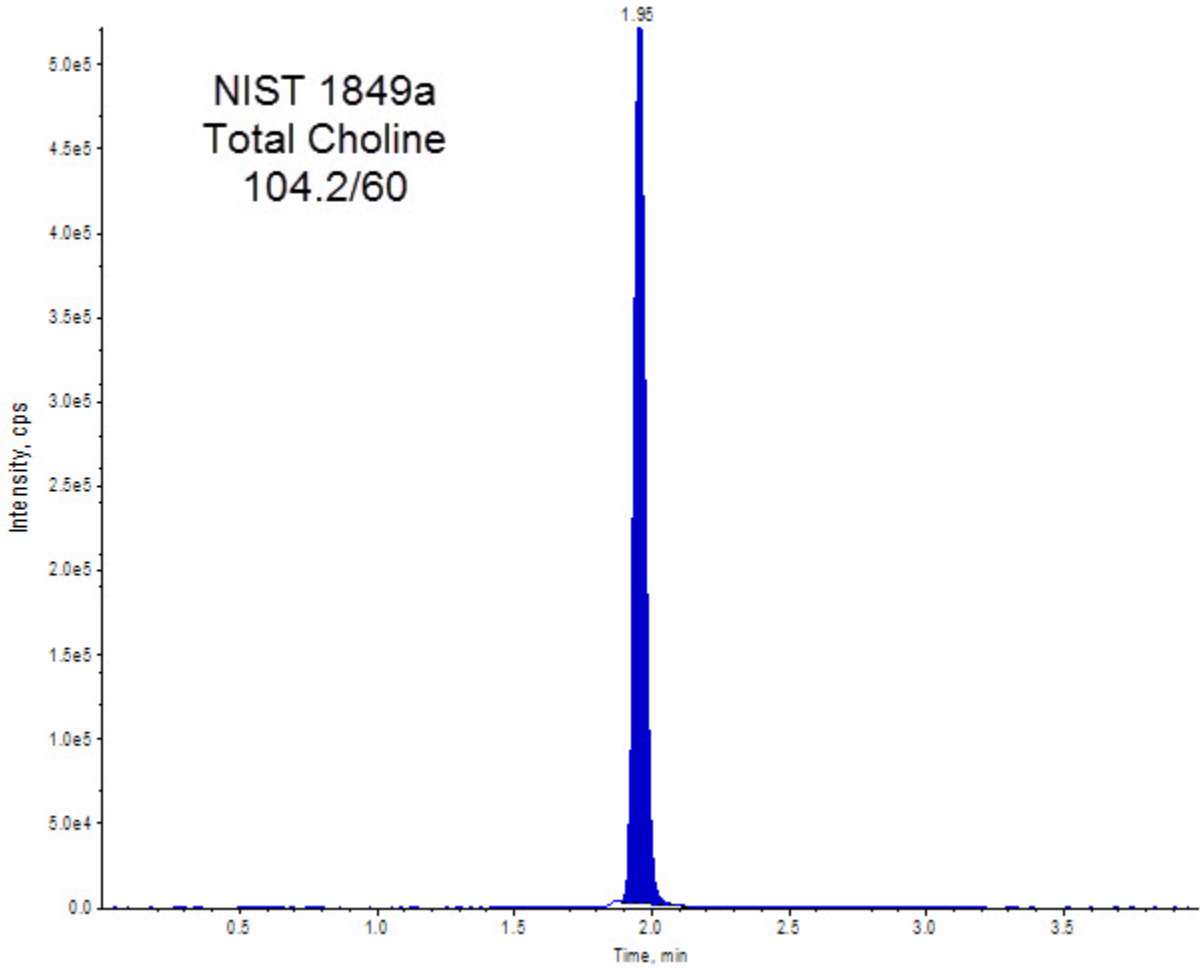

Figure 2015.10A. Extracted Ion Chromatogram (XIC) of Choline

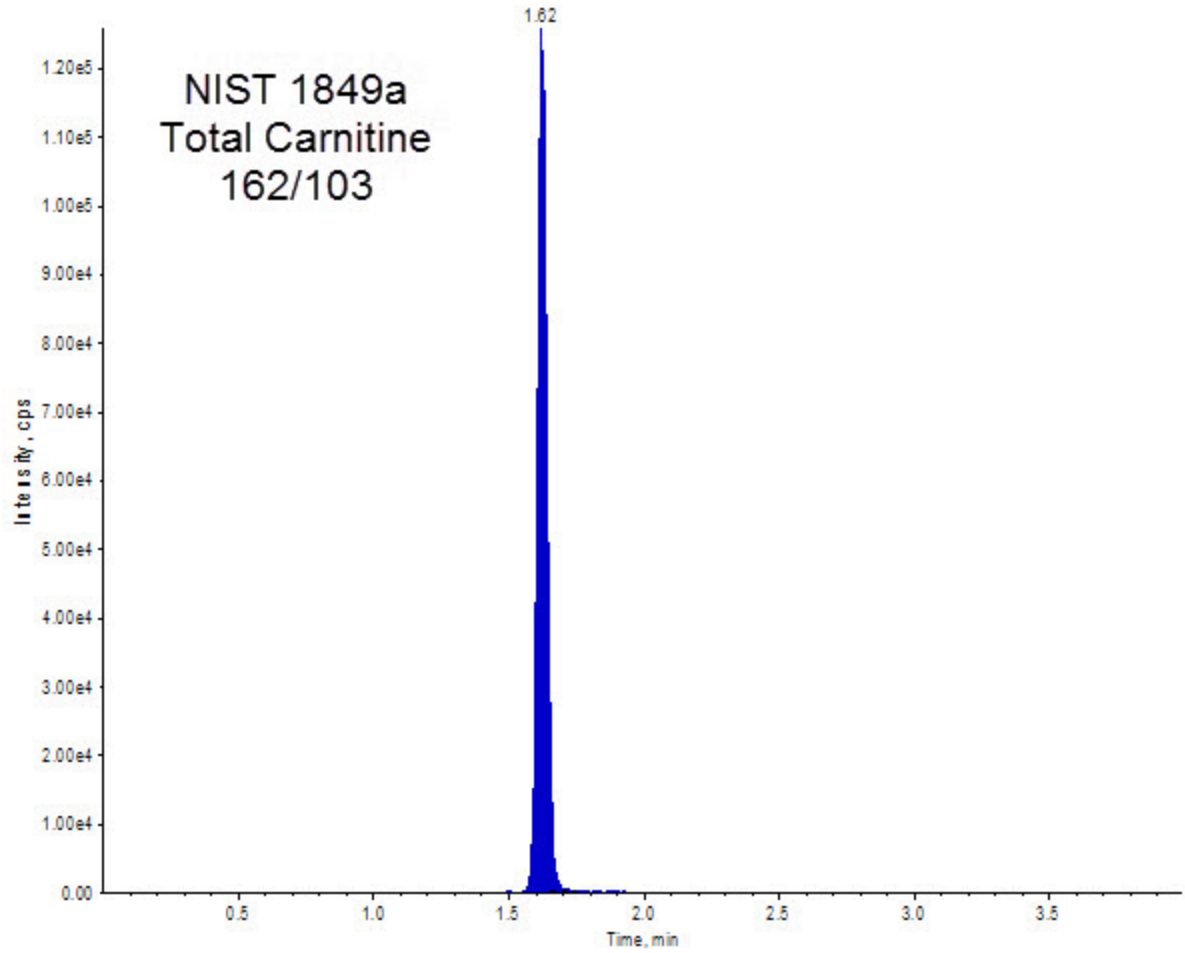

Figure 2015.10B. Extracted Ion Chromatogram (XIC) of Carnitine

Table 2015.10B. Compound Transitions

Compound	Use/Type	Transition (Q1/Q3)	Collision Energy (V)	Retention Time (min)
Carnitine	Quantitation	162.0/103.0	25	1.7
Carnitine-d3	Internal Standard	165.0/103.0	25	1.7
Carnitine	Confirmation	162.0/84.4	29	1.7
Carnitine	Confirmation	162.0/59.1	27	1.7
Choline	Quantitation	104.2/60.0	25	2.1
Choline-d4	Internal Standard	108.2/60.0	25	2.1
Choline	Confirmation	104.2/45.2	25	2.1

INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Free and Total Choline and Carnitine in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS): Single-Laboratory Validation, First Action 2015.10

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Analytical methods for the analysis of both L-carnitine and choline are needed for reliable and accurate determination in infant formula and adult/pediatric nutritional formula. These compounds are different in how they are utilized by the human body, but are structurally similar. L-carnitine and choline are quaternary ammonium compounds, enabling both to be retained under acidic conditions with strong cation exchange (SCX) chromatography. This method analyzes both compounds simultaneously as either the free forms or as a total amount that includes bound sources such as phosphatidylcholine or acetylcarnitine. The free analysis consists of water extraction and analysis by LC/MS/MS, while the total analysis consists of extraction by acid assisted microwave hydrolysis and analysis by LC/MS/MS. Calibration standards used for calculations are extracted with all samples in the batch. A single laboratory validation (SLV) was performed following the guidelines of the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) utilizing the kit of materials provided. The results achieved meet the requirements of SMPR 2012.010 and 2012.013 for L-carnitine and total choline, respectively.

An analytical method for the analysis of L-carnitine and total choline in infant formula (IF) and adult/pediatric nutritional formula is needed to meet the *Standard Method Performance Requirements* (SMPRs®) of the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN; 1, 2). There are currently other methods that can either quantify carnitine or total choline in IFs (3–5). We developed a method that can analyze these compounds simultaneously using strong cation-exchange (SCX) chromatography because both are quaternary ammonium compounds and can be retained under acidic conditions. This method can be used to quantify the free or total choline and carnitine content of a sample. The free portion is analyzed using water extraction, whereas the total extraction uses acid-assisted microwave hydrolysis. The total amount can include contribution from bound sources such as phosphatidylcholine or acetylcarnitine. Both extraction methods use LC tandem mass spectrometry (MS/MS) analysis with electrospray ionization (ESI). Calibration standards are included through each extraction procedure for greater assurance with quantification, and the acidic conditions of the total extraction allow direct injection after dilution with acetonitrile for fast analysis. A single-laboratory validation (SLV) was performed with this method using the SPIFAN SLV kit of materials. All requirements of SMPRs were met, except the ability to differentiate L- and D-carnitine. Verification for recovery of the bound forms of choline and carnitine commonly found in these matrixes was also completed.

Received June 1, 2015. Accepted by AK July 31, 2015.
This method was approved by the Expert Review Panel for Infant Formula and Adult Nutritionals as First Action.
The Expert Review Panel for Infant Formula and Adult Nutritionals invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
Corresponding author's e-mail: david.ellingson@covance.com
DOI: 10.5740/jaoacint.15-0144

AOAC Official Method 2015.10
Determination of Free and Total Choline and Carnitine in Infant Formula and Adult/Pediatric Nutritional Formula
Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)
First Action 2015

[Applicable to the determination of free and total choline and carnitine in infant formula and adult/pediatric nutritional formula.]

Caution: It is recommended that all preparation steps with nitric acid be performed within a fume hood, and the necessary personal protective equipment used when handling.

A. Principle

The method uses a water extraction for free analysis and acid-assisted microwave hydrolysis for total analysis. Both compounds are simultaneously analyzed and quantified by LC/MS/MS with ESI.

B. Apparatus

(a) *LC system*.—Prominence, Shimadzu (Kyoto, Japan) or equivalent.

(b) *MS/MS system*.—API 4000 with ESI, ABSciex (Framingham, MA) or equivalent.

(c) *Mass spectrometry software*.—Analyst (ABSciex) or equivalent.

(d) *Analytical column*.—Zorbax 300-SCX, 3.0 × 50 mm, 5 µm, Agilent (Santa Clara, CA) or equivalent.

(e) *Microwave*.—MARS6, CEM (Mathews, NC) or equivalent.

(f) *Microwave turntable, liner, and cap*.—MARSXpress, 55 mL PFA Teflon®, 40 position (CEM or equivalent).

(g) *Vortex mixer*.—VWR (West Chester, PA) or equivalent.

(h) *Analytical balances*.—Model CPA225D, Sartorius (Goettingen, Germany) or equivalent.

(i) *Horizontal shaker*.—Model 6010, Eberbach (Ann Arbor, MI) or equivalent.

(j) *Magnetic stir plate*.—Model PC-420D, Corning (Corning, NY) or equivalent.

(k) *Positive displacement pipets*.—Microman, various sizes, Gilson (Middleton, WI) or equivalent.

(l) *Repeater positive displacement pipet*.—Repeater Plus, Eppendorf (Hamburg, Germany) or equivalent.

(m) *Polypropylene tubes*.—Digitube, assorted sizes, SCP Science (Montreal, Canada) or equivalent.

(n) *Mobile phase containers*.—2 L, glass, VWR or equivalent.

(o) *Syringe filters*.—0.45 µm PTFE and hydrophilic polypropylene (GHP), Pall (Plano, TX) or equivalent.

(p) *Disposable syringes*.—3 mL, BD Biosciences (Franklin Lakes, NJ) or equivalent.

(q) *Graduated cylinders*.—Assorted sizes, VWR or equivalent.

(r) *Magnetic stir bars*.—7.9 × 50 mm, VWR or equivalent.

(s) *Autosampler vials/caps*.—1.5 mL silanized crimp top, VWR or equivalent.

(t) *Microcentrifuge tubes*.—1.5 mL polypropylene, VWR or equivalent.

(u) *Bottle top dispenser*.—5 mL acid resistant, Brand (Essex, CT) or equivalent.

(v) *Desiccator*.—Glass, VWR or equivalent.

Note: Nonspecific binding can occur with these analytes when using glassware, so plasticware should be used at all times for standard/sample preparation. All laboratory plasticware should be single-use whenever possible. Positive displacement pipets are also mandatory for pipeting to avoid contamination and for accuracy with organic solvents.

C. Chemicals and Reagents

(a) *Water*.—Optima MS grade, Thermo Fisher Scientific (Waltham, MA) or equivalent.

(b) *Acetonitrile*.—Optima MS grade, Thermo Fisher Scientific or equivalent.

(c) *Ammonium formate*.—Optima MS grade, Thermo Fisher Scientific or equivalent.

(d) *Formic acid*.—Optima MS grade, Thermo Fisher Scientific or equivalent.

(e) *Nitric acid*.—70% (w/w), ACS grade, Avantor (Center Valley, PA) or equivalent.

(f) *Isopropanol*.—Optima MS grade, Thermo Fisher Scientific or equivalent.

(g) *Desiccant*.—VWR or equivalent.

(h) *Reference standard*.—L-Carnitine, USP (Rockville, MD) or equivalent.

(i) *Reference standard*.—Choline bitartrate, TCI (Tokyo, Japan) or equivalent.

(j) *Reference internal standard*.—L-Carnitine-d_3 HCl, CDN Isotopes (Pointe Claire, Québec, Canada or equivalent).

(k) *Reference internal standard*.—Choline-1,1,2,2-d_4 chloride (CDN Isotopes or equivalent).

Note: All use of water in this method must be high-purity MS-grade water.

D. Mobile Phase Preparation

Mobile phase A [5 mM ammonium formate in 50 + 50 (v/v) water–acetonitrile with 0.2% formic acid] was prepared by weighing 0.63 g ammonium formate into a 1 L graduated cylinder. Water was added along with a stir bar and mixed to dissolve before diluting to volume with water. The solution was transferred to a 2 L mobile phase container along with 1 L acetonitrile, 4 mL formic acid, a stir bar, and then thoroughly mixed. Mobile phase B [30 mM ammonium formate in 50 + 50 (v/v) water–acetonitrile with 0.2% formic acid] was prepared by weighing 3.78 g ammonium formate into a 1 L graduated cylinder. Water was added along with a stir bar and mixed to dissolve before diluting to volume with water. The solution was transferred to a 2 L mobile phase container along with 1 L acetonitrile, 4 mL formic acid, a stir bar, and then thoroughly mixed. Mobile phase B was also used for the rinse solutions in the autosampler.

E. Preparation of Standard Solutions

The carnitine stock standard was prepared at a concentration of 25 mg/mL by weighing 0.25 g L-carnitine into a 20 mL polypropylene tube followed by 10 mL water to dissolve. The purity of L-carnitine from the Certificate of Analysis (CoA) and moisture determined by Karl Fischer titration immediately at the time of weighing was used to calculate the final concentration of carnitine. The choline stock standard was prepared at a concentration of 25 mg/mL choline by weighing 0.62 g choline bitartrate into a 20 mL polypropylene tube followed by 10 mL water to dissolve. The purity of choline bitartrate from the CoA along with a molecular weight conversion from choline bitartrate to choline of 0.41133, was used to calculate the final concentration of choline. Intermediate working standards were prepared at concentrations of 10, 20, 500, 2000, 4000, and 5000 µg/mL for each analyte using both the stock and higher concentration intermediate working standard solutions using appropriate volumes into 20 mL polypropylene tubes with water as the diluent. All stock and intermediate standard solutions were stable for 2 months when stored at 5 ± 3°C and protected from light. Aliquots of the intermediate working standards were treated through the sample analysis, so the concentrations used for the calibration curves for both free and total analyses were the same numerical values as the intermediate working standards but in ng/mL. Internal stock standards were prepared at a concentration of 2 mg/mL by weighing 25 mg L-carnitine-d_3 and 35 mg choline-1,1,2,2-d_4 into separate 20 mL polypropylene tubes. A volume of 10 mL water was added to each to dissolve, and then both solutions quantitatively transferred to a 100 mL polypropylene tube and diluted to volume with water to prepare an intermediate solution at 200 µg/mL. The purity from the CoA was used to calculate the final concentration of each internal standard. Stability of these solutions was monitored while being stored at 5 ± 3°C and protected from light.

F. Sample Preparation

Powder IF and adult nutritionals were reconstituted by weighing 25 g and diluting with water to a final weight of 225 g.

Viscous ready-to-feed (RTF) products that were being analyzed for total choline and carnitine were prediluted by weighing 1.0 g and diluting with water to a final weight of 5.0 g.

(a) *Free choline and carnitine*.—Samples were prepared by weighing 1.0 g of reconstituted product into a 50 mL polypropylene tube. Six additional tubes were designated for the working standards along with two tubes for the reagent blank and reagent blank + internal standard to monitor any interference or carryover. The working standards, reagent blank, and reagent blank + internal standard were included with each free analysis and treated the same as samples through the sample preparation. The working standard tubes received 50 µL of the appropriate intermediate working standard level. All tubes except the reagent blank received 50 µL of the intermediate internal standard solution. The tubes were diluted to 25 mL with water and thoroughly mixed on a horizontal shaker. The reagent blank + internal standard solution was used as the diluent if dilutions were needed. A 0.5 mL aliquot the sample solution was mixed with 0.5 mL of acetonitrile in a microcentrifuge tube, and then filtered through a 0.45 µm GHP syringe filter into a silanized injection vial. Aliquots of 0.5 mL of the working standard and reagent blank solutions were mixed with 0.5 mL acetonitrile directly in the silanized injection vials.

(b) *Total choline and carnitine*.—Samples were prepared by weighing 1.0 g of reconstituted or diluted product into a 55 mL MARSXpress liner. Six additional liners were designated for the working standards along with two liners for the reagent blank and reagent blank + internal standard to monitor any interference or carryover. The working standards, reagent blank, and reagent blank + internal standard were included with each total analysis and treated the same as samples through the sample preparation. Liners designated for the working standards received 50 µL of the appropriate intermediate working standard level. All liners except the reagent blank received 50 µL of the intermediate internal standard solution. A 5 mL volume of water followed by 2.5 mL of 70% (w/w) nitric acid delivered with a bottle top dispenser were then added to each liner, capped, and vortexed to mix. The microwave program used was a ramp to temperature of 120°C over 10 min, followed by a 40 min hold at a power of 1000 W, ending in a cool down (6). The contents of the vessels were transferred into 50 mL polypropylene tubes with water and diluted to a volume of 25 mL with water. A 0.5 mL aliquot of the sample solution was mixed with 0.5 mL acetonitrile in a microcentrifuge tube, and then filtered through a 0.45 µm PTFE syringe filter into a silanized injection vial. Aliquots of 0.5 mL of the working standard and reagent blank solutions were mixed with 0.5 mL of acetonitrile directly in the silanized injection vials.

G. LC/MS/MS Parameters

A Shimadzu Prominence LC system equipped with an Agilent Zorbax 300-SCX column (3.0 × 50 mm, 5 µm) was used. A flow rate of 1.0 mL/min was maintained over the 4.2 min total run time. The mobile phase conditions were 100% mobile phase A until 1.0 min, ramped to 100% mobile phase B by 1.5 min, and ramped back to 100% phase A by 3.0 min. A column temperature of 40°C, and an autosampler temperature of 5°C was maintained. A 1 µL injection was used. Autosampler rinse settings were adjusted to eliminate carryover as much as possible. An ABSciex API 4000 mass spectrometer with positive ESI was used in multiple reaction monitoring (MRM) mode. The MS/MS overall settings used are described in Table **2015.10A**. The MS/MS settings may need to be modified except for ionization, mode, and gas types to obtain optimum chromatography and sensitivity. Figures **2015.10A** and **2015.10B** show typical extracted ion chromatograms (XICs) from National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) 1849a for choline and carnitine.

H. Quantification and Confirmation

The quantification of choline and carnitine was accomplished by the generation of calibration curves using the peak area ratio of the chosen transition (Table **2015.10B**) versus the corresponding deuterated internal standards. Least-squares regression analysis using a linear model with $1/x^2$ weighting was used for both analytes. Confirmation was achieved through the analysis of ion ratios between samples and reference standards for at least one additional transition listed in the table. The concentration of each analyte in a sample was calculated by the following equation:

$$R = \frac{C \times V \times D}{S} \times \frac{100}{10^6\ ng/mg}$$

Table 2015.10A. MS/MS settings

Ionization	Positive ion electrospray (ESI+)
Mode	MRM
Ion spray voltage	1000 V
Turbo ion spray temp.	550°C
Declustering potential	120 V
Dwell time	100 msec
Entrance potential	10 V
Collision cell exit potential	25 V
Collision gas	Nitrogen, 5 psig
Curtain gas	Nitrogen, 20 psig
Nebulizing gas (gas 1)	Nitrogen, 60 psig
Auxiliary gas (gas 2)	Nitrogen, 60 psig
Needle position	*Y* = 5 mm, *X* = 5 mm

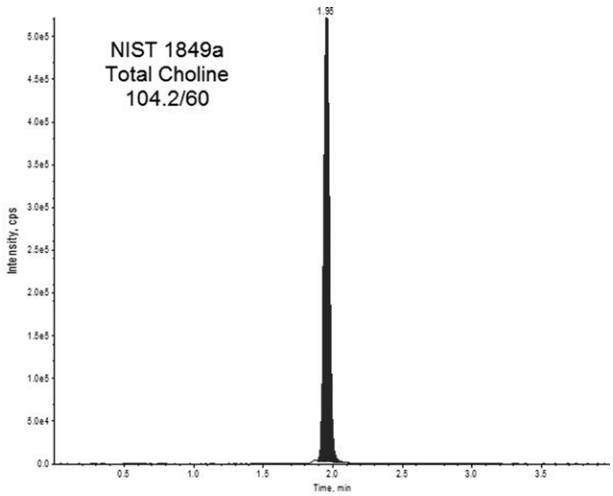

Figure 2015.10A Extracted ion chromatogram (XIC) of choline.

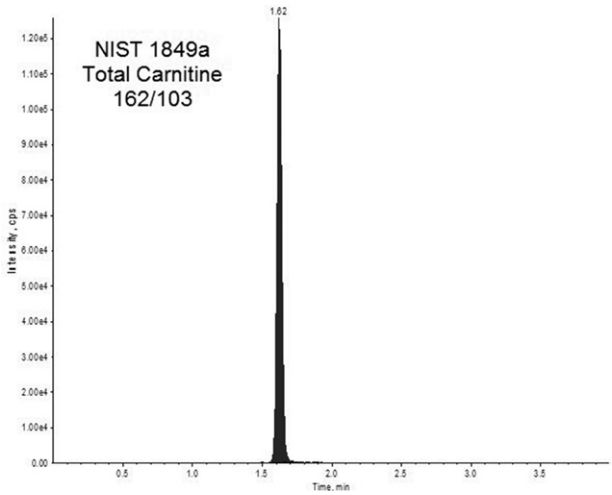

Figure 2015.10B. Extracted ion chromatogram (XIC) of carnitine.

Table 2015.10B. Compound transitions

Compound	Use/type	Transition (Q1/Q3)	Collision energy, V	Retention time, min
Carnitine	Quantitation	162.0/103.0	25	1.7
Carnitine-d3	Internal standard	165.0/103.0	25	1.7
Carnitine	Confirmation	162.0/84.4	29	1.7
Carnitine	Confirmation	162.0/59.1	27	1.7
Choline	Quantitation	104.2/60.0	25	2.1
Choline-d4	Internal standard	108.2/60.0	25	2.1
Choline	Confirmation	104.2/45.2	25	2.1

where R = results expressed in mg/100 g, C = concentration of the analyte in the injected solution in ng/mL, V = volume of the initial extract in mL, S = sample weight in grams, and D = dilution factor, the inverse of any dilution made. All results were calculated on a RTF or reconstituted basis of 25 g diluted to 225 g with water, except for SRM 1849a that was calculated back to powder basis.

Results and Discussion

Linearity was assessed from the percent deviation from the theoretical concentration across the working standard range (Table 1). The results from the validation showed an overall repeatability for free and total choline of 1.9 and 2.3% RSD_r, whereas the overall intermediate precision obtained for free and total choline was 2.4 and 2.7% RSD_{INT}, respectively (Table 2). Free and total carnitine had an overall repeatability of 2.9 and 2.7% RSD_r, whereas the overall intermediate precision obtained for free and total carnitine was 3.3 and 3.1% RSD_{INT},

Table 1. Linearity[a]

	Percent deviation from theoretical concn, ng/mL						
Compound	10	20	500	2000	4000	5000	Mean
Carnitine	2.9	3.0	2.0	2.4	1.2	2.0	2.2
Choline	2.5	4.1	1.4	1.1	1.0	1.7	1.9

[a] Average from three analyses of duplicates at each working standard level.

Table 2. Choline precision summary[a]

	Free choline			Total choline		
Sample	Mean concn, mg/100 g	RSD_r, %	RSD_{INT}, %	Mean concn, mg/100 g	RSD_r, %	RSD_{INT}, %
SRM 1849a[b,c]	81.3	2.1	2.3	105	2.1	1.8
AN powder milk protein-based	0.364[d]	1.8[d,e]	2.7[d,f]	4.11	2.3[g]	2.8[f]
IF hydrolyzed milk-based	14.4	1.7	2.7	18.8	2.1	3.5
IF hydrolyzed soy-based	17.8	1.7	2.7	17.8	2.0	2.9
AN powder low fat	15.5	1.6	3.5	18.1	2.4[h]	2.8
Child formula powder	4.61	1.0	1.7	5.70	2.3	2.6
IF elemental powder	8.11	1.1	2.2	8.68	2.2	2.8
IF powder milk-based	13.8	2.2	2.5	17.4	2.1[i]	3.3[f]
IF powder soy-based	14.9	1.1	2.1	20.2	2.6	4.0
IF RTF milk-based	12.6	3.1[i]	2.7[g]	21.4	2.8	2.5
AN RTF high protein	42.8	2.7	2.6	49.3	1.6	1.5
AN RTF high fat	48.1	1.4	1.5	53.2	1.2	1.5
Child formula powder placebo	0.205[d]	2.2[d]	4.0[d]	1.25[d]	2.7[d]	2.2[d]
IF elemental powder placebo	0.118[d]	6.4[d]	6.4[d]	0.651[d]	1.8[d]	3.0[d]
AN RTF high protein placebo	0.491[d]	1.7[d]	1.9[d]	6.03	3.4	3.2
AN RTF high fat placebo	0.522[d]	2.8[d]	2.8[d]	5.10	2.7	2.5
IF RTF milk-based placebo	5.42	1.6	2.4	13.2	2.0	2.2
Overall		1.9	2.4		2.3	2.7

[a] Samples were run in triplicate across 4 days, n = 12. Deviation is footnoted due to outliers by Grubbs' test at a 95% confidence interval, loss of sample during preparation, or failure to prepare in triplicate. Twelve data points were still obtained for all samples.

[b] Free choline information mass fraction value of 79.8 mg/100 g.

[c] Total choline certified mass fraction mean and range of 109 mg/100 g and 98.0–120 mg/100 g, respectively.

[d] Results below the SMPR-required LOQ of 2.0 mg/100 g and are not included in the calculations.

[e] Three days of duplicates used for calculation.

[f] Calculated across 5 days.

[g] Two days of duplicates and 1 day of four replicates used for calculation.

[h] One day of duplicates and 1 day of four replicates used for calculation.

[i] One day of duplicates used for calculation.

Table 3. Carnitine precision summary[a]

	Free carnitine			Total carnitine		
Sample	Mean concn, mg/100 g	RSD_r, %	RSD_{INT}, %	Mean concn, mg/100 g	RSD_r, %	RSD_{INT}, %
SRM 1849a[b]	13.4	2.0	2.2	15.3	2.1	1.8
AN powder milk protein-based	<0.05[c]	NA[d]	NA	<0.05	NA	NA
IF hydrolyzed milk-based	0.909	1.8	2.1	1.21	4.4	4.8
IF hydrolyzed soy-based	1.05	1.8	3.0	1.04	1.6	2.2
AN powder low fat	<0.05	NA	NA	<0.05	NA	NA
Child formula powder	5.64	3.0	3.2	5.76	2.5	2.2
IF elemental powder	1.62	3.8	4.1	1.63	2.0	2.5
IF powder milk-based	1.59	2.1	2.1	1.82	2.7	4.7
IF powder soy-based	0.942	3.9	3.6	0.948	4.6	5.0
IF RTF milk-based	2.68	2.7[e]	3.5[f]	2.78	2.3	2.9
AN RTF high protein	15.5	3.6	3.9	15.7	1.9	1.7
AN RTF high fat	21.9	3.3	3.8	22.3	1.3	1.5
Child formula powder placebo	<0.05	NA	NA	<0.05	NA	NA
IF elemental powder placebo	<0.05	NA	NA	<0.05	NA	NA
AN RTF high protein placebo	<0.05	NA	NA	<0.05	NA	NA
AN RTF high fat placebo	<0.05	NA	NA	<0.05	NA	NA
IF RTF milk-based placebo	2.67	2.3	3.4	2.67	1.9	1.8
Overall		2.9	3.3		2.7	3.1

[a] Samples were run in triplicate across 4 days, n = 12. Deviation is footnoted due to outliers by Grubbs' test at a 95% confidence interval, loss of sample during preparation, or failure to prepare in triplicate. Twelve data points were still obtained for all samples.

[b] Free carnitine certified mass fraction mean and range of 13.6 mg/100 g and 12.2–15.0 mg/100 g, respectively.

[c] Results below the SMPR-required LOQ of 0.16 mg/100 g and not included in the calculations.

[d] NA = Not applicable.

[e] One day of duplicates used for calculation.

[f] Calculated across 5 days.

Table 4. Recovery analysis for choline[a]

	Free choline		Total choline	
Sample	50% Overspike, %	100% Overspike, %	50% Overspike, %	100% Overspike, %
IF powder soy-based	97.6	97.0	98.4	99.6
AN RTF high protein	100.1	98.3	98.3	98.6
AN RTF high fat	NA[b]	98.0[c]	NA	99.9[c,d]
IF RTF milk-based placebo	101.7	99.9	98.8	96.8
IF elemental powder placebo	95.9[e]	NA	99.3[e]	NA

[a] Samples were run in duplicate over three days at each level, n = 6.

[b] NA = Not applicable.

[c] Samples were spiked at an amount that would achieve the upper range (250 mg/100 g) of the SMPR for choline.

[d] n = 5, due to loss of sample during a day of analysis.

[e] Samples were spiked at an amount that would achieve the LOQ (2 mg/100 g) of the SMPR for choline.

Table 5. Recovery analysis for carnitine[a]

	Free carnitine		Total carnitine	
Sample	50% Overspike, %	100% Overspike, %	50% Overspike, %	100% Overspike, %
IF powder soy-based	99.3	100.2	98.7	99.7
AN RTF high protein	102.4	103.6	101.5	102.7
IF RTF milk-based placebo	99.2	99.8	101.4	99.9
IF elemental powder placebo	102.5[b]	NA[c]	101.6[b]	NA

[a] Samples were run in duplicate over 3 days at each level, n = 6.

[b] Samples were spiked at an amount that would achieve the LOQ (0.16 mg/100 g) of the SMPR for carnitine.

[c] NA = Not applicable.

respectively (Table 3). The average results obtained from the analysis of NIST SRM 1849a were within the certified ranges or close to the information mass fraction value. Only an information mass fraction value is given for free choline, although there is currently nothing provided for total carnitine from NIST. The average total recoveries (endogenous + added) shown in Tables 4 and 5 for all matrixes tested from LOQ to the upper ranges required in the SMPR were 95.9–103.6%. Analysis of bound sources of carnitine and choline analyzed in duplicate over 3 days gave average recoveries of 104.6% for acetylcholine, 96.7% for phosphatidylcholine, and 104.1% for acetylcarnitine. The level of detection (LOD) was determined by the mean baseline noise + (3 × SD) from 10 blank replicates, and then adjusted with the default weights and dilutions used in the method. An LOD of 0.0034 mg/100 g for both free and total carnitine analyses and an LOD of 0.0047 mg/100 g for both free and total choline analyses were achieved. An LOQ of 0.05 mg/100 g was obtained for both free and total choline and carnitine. The LOQ was calculated from the lowest working standard concentration through the default weights and dilutions used in the method. The analytical range of the method is from the stated LOQ to 250 mg/100 g choline and 20 mg/100 g carnitine and is supported by the data collected from the precision and accuracy experiments. This method meets all requirements of AOAC SMPRs 2012.010 and 2012.013 for L-carnitine and total choline, respectively; the only exception is an inability to distinguish between L- and D-carnitine with this method.

References

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AOAC Official Method 2015.11
Chondroitin Sulfate Content in Raw Materials and Dietary Supplements
High-Performance Liquid Chromatography with Ultraviolet Detection After Enzymatic Hydrolysis
First Action 2015

A. Materials

Chondroitin sulfate (CS) raw materials from bovine trachea, porcine skin/cartilage, and shark cartilage, and CS control material from bovine trachea were obtained from Bioiberica (Barcelona, Spain). Dietary supplement products containing CS (hard-shell capsules, tablets, chewables, softgels, and liquids) were obtained from commercial suppliers. Descriptions of the dietary supplement products used in the study are presented in Table **2015.11A**.

B. Apparatus

(**a**) *LC system*.—Beckman 126 dual high pressure mixing pumps (Beckman Coulter, Fullerton, CA, USA), 168 diode array UV detector, 507e autosampler, and 32 Karat software.

(**b**) *Operating conditions*.—Mobile phase flow rate, 1.1 mL/min; column temperature, ambient; injection volume, 30 µL; and detection, 240 nm.

(**c**) *LC column*.—Phenomenex Synergi Polar-RP, 4.6 × 150 mm, 4 µm particle size (Phenomenex, Torrance, CA, USA).

(**d**) *Analytical balance*.—Accu-124 (Fisher Scientific, Pittsburgh, PA, USA), ±0.01 mg readability.

(**e**) *Ultrasonic bath*.—Model FS60H (Fisher Scientific).

(**f**) *pH meter*.—Model pH 500 (Oakton, Vernon Hills, IL, USA), ±0.01 pH unit readability.

(**g**) *Dry block heater*.—Isotemp Dry Bath Incubator (Fisher Scientific), maintained at 37°C.

(**h**) *LC injection vials*.—2 mL, with caps and Teflon-coated septa.

(**i**) *Limited volume inserts*.—200 µL, for LC vials.

(**j**) *Syringes*.—25, 100, and 500 µL Luer-Lok.

C. Reagents

Note: Chemicals from other suppliers meeting the specifications may also be used.

(**a**) *Solvents*.—Acetonitrile, LC grade; water, LC grade; hydrochloric acid, concentrated, ACS reagent grade.

(**b**) *Tetrabutylammonium bisulfate*.—Minimum 99.0% (Fluka, St. Louis, MO, USA; http://www.sigma-aldrich.com; Cat. No. 86868).

(**c**) *Tris-(hydroxymethyl)aminomethane (TRIS)*.—Sigma (St. Louis, MO, USA), Cat. No. T-1503.

(**d**) *Sodium acetate*.—Anhydrous (Sigma, Cat. No. S-8750).

(**e**) *Acetic acid*.—Glacial (Sigma, Cat. No. A-0808).

(**f**) *Sodium chloride*.—ACS reagent grade.

(**g**) *Bovine serum albumin*.—1x Crystallized, 97% (Sigma, Cat. No. A-4378).

(**h**) *Chondroitinase AC II*.—Five units (Seikagaku America/Associates of Cape Cod, East Falmouth, MA, USA; Cat. No. 100335-1A, http://www.acciusa.com).

(**i**) *Mobile phase A*.—Weigh 340 mg tetrabutylammonium bisulfate and transfer into a 1000 mL volumetric flask. Dissolve and dilute to volume with water. Degas.

(**j**) *Mobile phase B*.—Weigh 340 mg tetrabutylammonium bisulfate and dissolve in 330 mL deionized (DI) water. Add acetonitrile to 1000 mL. Sonicate and filter.

(**k**) *0.12 M HCl*.—Carefully add 1 mL concentrated HCl to 99 mL water and mix well.

(**l**) *6 M HCl*.—Carefully add 50 mL concentrated HCl to 50 mL water and mix well.

(**m**) *TRIS buffer solution*.—Dissolve 3 g TRIS, 2.4 g sodium acetate, 1.46 g sodium chloride, and 50 mg crystalline bovine serum albumin in 100 mL of 0.12 M HCl. Adjust pH to 7.3 with 6 M HCl.

(**n**) *Enzyme solution*.—Dissolve 5 units of chondroitinase ACII enzyme in 0.5 mL water. Store at <0°C when not in use.

(**o**) *Dilution solution*.—Prepare at least 20 mL of a solution containing 80% mobile phase A and 20% mobile phase B.

(**p**) *Reference standards*.—*See* Table **2015.11B**. Purities were obtained from the supplier's certificate of analysis. These purities were determined by chromatographic purity, water content, and residual solvent content. No independent confirmation of the purity was performed.

D. Preparation of Test Solutions

(**a**) *Preparation of standard solutions*.—Accurately weigh about 2 mg ΔDi-0S and 10 mg each of ΔDi-4S and ΔDi-6S (Table **2015.11B**), and transfer into a 50 mL volumetric flask. Dissolve and dilute to volume with water. This is the stock instrument calibration solution.

(**b**) *Instrument calibration solutions*.—Prepare serial dilutions of the stock instrument calibration solution in water at concentrations of about 2, 8, 20, 40, and 100 µg/mL ΔDi-4S and ΔDi-6S, and 0.4, 1.6, 4, 8, and 20 µg/mL ΔDi-0S. A possible dilution scheme is shown in Table **2015.11C**.

Table 2015.11A. Materials

Material No.	Type	Composition	Claim
1	Raw material	CS from bovine trachea	Pure
2	Capsules	CS	250 mg CS/capsule
3	Chewables	CS + glucosamine, ascorbic acid	125 mg CS/wafer
4	Tablets	CS + glucosamine sulfate (from marine source)	400 mg CS/tablet
5	Softgels	CS + glucosamine	200 mg CS/softgel
6	Raw material	CS from porcine	Pure
7	Raw material	CS from shark cartilage	Pure
8	Tablets	CS + ascorbic acid, manganese, herbs	200 mg CS/tablet
9	Liquid	CS + glucosamine, MSM, ascorbic acid, manganese	800 mg CS/29.57 mL

Table 2015.11B. Reference standards

Name	Abbreviation	Supplier
2-Acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-D-galactose	ΔDi-0S	Sigma
2-Acetamido-2-deoxy-3-*O*(β-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose	ΔDi-4S	Sigma
2-Acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose	ΔDi-6S	Sigma
2-Acetamido-2-deoxy-3-*O*-(2-*O*-sulfo-β-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose	ΔDi-di(2,6)S	ICN[a]
2-Acetamido-2-deoxy-3-*O*-(β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose	ΔDi-di(4,6)S	ICN
2-Acetamido-2-deoxy-3-*O*-(2-O-sulfo-β-D-gluco-4-enepyranosyluronic acid)-4,6-di-*O*-sulfo-D-galactose	ΔDi-tri(2,4,6)S	ICN

[a] Now MP Biomedicals (Solon, OH, USA).

(**c**) *CS control solution*.—Accurately weigh about 100 mg CS control sample into a 50 mL volumetric flask. Add about 30 mL water, and sonicate until the sample is completely dissolved (about 15 min). Dilute to volume with water and mix well. Label Control Solution 1.

(**d**) *Sample test solutions*.—(*1*) *Raw materials*.—Accurately weigh about 200 mg CS raw material into a 100 mL volumetric flask. Add 60 mL water, and sonicate until the sample is completely dissolved (about 15 min). Dilute to volume with water and mix well. Label Test Solution 1.

(*2*) *Tablets*.—Determine the average tablet weight by weighing 20 tablets and calculating the average weight of one tablet. Grind the 20 tablets to a powder and mix. Accurately weigh a test portion containing the equivalent of about 200 mg CS into a 100 mL volumetric flask. Add about 60 mL water and sonicate for 15 min. Dilute to volume with water and mix thoroughly. Filter ca 1–2 mL Test Solution 1 through a 0.2 µm PTFE syringe filter. Label Test Solution 1.

(*3*) *Capsules*.—Determine the average capsule content weight by weighing 20 capsules. Record the weight. Empty and combine the capsule contents. Thoroughly clean the capsule shells using a swab and/or compressed air. Weigh and record the weight of the empty capsule shells:

$$\text{Average capsule fill weight, g} = \text{g} = \frac{C - S}{20}$$

where C = total weight of 20 capsules and S = total weight of 20 capsule shells. Proceed as directed in (**d**)(*2*) for tablets.

(*4*) *Liquid formulations*.—Thoroughly mix the sample. Accurately weigh an amount of test portion containing the equivalent of about 200 mg into a 100 mL volumetric flask. Dissolve in and dilute to volume with water. Mix thoroughly. Label Test Solution 1.

(**e**) *Enzymatic hydrolysis of control solution and test solution*.—Pipet 20 µL TRIS buffer solution, 30 µL enzyme solution, and 20 µL Control Solution 1 or Test Solution 1 into a 2 mL LC injection vial with a 200 µL insert. Place the vial in a 37°C dry bath or water bath for 3 h. Allow to cool room temperature. Using an automatic pipettor or gas-tight syringe, carefully transfer the solution into an LC vial. Rinse the 200 µL insert with exactly 100 µL mobile phase A using a calibrated automatic pipettor or gas-tight syringe, and quantitatively transfer this into the LC vial. Dilute to 1.00 mL by adding 830 µL mobile phase A to the LC vial. Mix well. Label Control Solution 2 or Test Solution 2–Treated.

Table 2015.11C. Preparation of instrument calibration solutions

Calibration solutions	Volume of stock pipetted, mL	Final volume (flask size), mL
1	25	50
2	10	50
3	5	50
4	2	50
5	1	100

E. Determination

(**a**) *Mobile phase gradient program*.—Elute the analytes with the linear gradient program of mobile phases A and B shown in Table **2015.11D**.

(**b**) *System suitability tests*.—Equilibrate the LC system with the mobile phases for at least 30 min until a stable baseline is obtained. Inject each of the five instrument calibration solutions. Use linear regression to determine the slopes, y-intercepts, and correlation coefficient (r^2) of the calibration lines for ΔDi-0S, ΔDi-4S, and ΔDi-6S. The correlation coefficient of the calibration line for each component must be >0.998 (for Di-0S >0.995). The tailing factor for all the components in the linearity standards must be between 0.80 and 1.5. Inject Control Solution 2–Treated and calculate the total amount of CS in the control material, **F**(**a**)–(**h**). The recovery should be within ±3% of the specification.

(**c**) *Injection*.—Make single injections of each standard and test solution. After every 20 sample injections, and after all of the sample injections are completed, make a single injection of each standard solution.

(**d**) *Retention times*.—The approximate retention times for each analyte are presented in Figure **2015.11A**.

(**e**) *Chromatograms*.—Representative standard and sample chromatograms are presented in Figures **2015.11A–C**.

Table 2015.11D. Linear mobile phase gradient[a]

Time, min	Mobile phase A, %	Mobile phase B, %
0	80	20
0–7.0	35	65
7.0–12.0	35	65
12–12.5	80	20

[a] The column should be reequilibrated at the starting mobile phase conditions for at least 10 min after each injection.

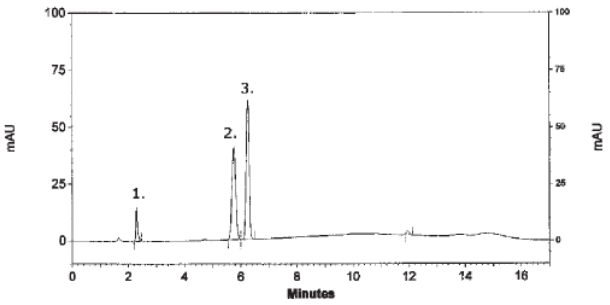

Figure 2015.11A. Stock standard solution chromatogram. Peak assignments and approximate retention times: (*1*) ΔDi-0S (2.5 min), (*2*) ΔDi-6S (5.9 min), (*3*) ΔDi-4S (6.3). Not shown: ΔDi-di(2,4)S (9.6 min), ΔDi-di(2.6)S (9.9 min), and ΔDi-tri(2,4,6)S (12.0 min).

F. Calculations

(**a**) The amount of ΔDi-0S in µg/g, representing unsulfated CS in the sample, is calculated as follows:

$$\frac{P_0 - b_0}{m_0} \times \frac{V}{W} \times D$$

where P_0 = peak area of ΔDi-0S in sample chromatogram; b_0 = y-intercept of calibration curve for disaccharide ΔDi-0S; m_0 = slope of calibration curve for disaccharide ΔDi-0S; V = volume of Test Solution 1 = 100 mL; W = sample weight, in g; and D = dilution factor = 50.

(**b**) The amount of ΔDi-4S in µg/g, representing CSA in the sample, is calculated as follows:

$$\frac{P_4 - b_4}{m_4} \times \frac{V}{W} \times D$$

where P_4 = peak area of ΔDi-4S in sample chromatogram; b_4 = y-intercept of calibration curve for disaccharide ΔDi-4S; m_4 = slope of calibration curve for disaccharide ΔDi-4S; V = volume of Test Solution 1 = 100 mL; W = sample weight, in g; and D = dilution factor = 50.

(**c**) The amount of ΔDi-6S in µg/g, representing CSC in the sample, is calculated as follows:

$$\frac{P_6 - b_6}{m_6} \times \frac{V}{W} \times D$$

where P_6 = peak area of ΔDi-6S in sample chromatogram; b_6 = y-intercept of calibration curve for disaccharide ΔDi-6S; m_6 = slope of calibration curve for disaccharide ΔDi-6S; V = volume of Test Solution 1 = 100 mL; W = sample weight, in g; and D = dilution factor = 50.

(**d**) The amount of ΔDi-di(2,6)S in µg/g in the sample is calculated as follows:

$$\frac{P_{2,6} - b_6}{m_6} \times \frac{V}{W} \times D \times F$$

where $P_{2,6}$ = peak area of ΔDi-di(2,6)S in sample chromatogram; b_6 = y-intercept of calibration curve for disaccharide ΔDi-6S; m_6 = slope of calibration curve for disaccharide ΔDi-6S; V = volume of Test Solution 1 = 100 mL; W = sample weight, in g; D = dilution factor = 50; and F = molecular weight conversion between ΔDi-6S and ΔDi-di(2,6)S = 1.190.

(**e**) The amount of ΔDi-di(4,6)S in µg/g in the sample is calculated as follows:

$$\frac{P_{4,6} - b_6}{m_6} \times \frac{V}{W} \times D \times F$$

where $P_{2,6}$ = peak area of ΔDi-di(4,6)S in sample chromatogram; b_6 = y-intercept of calibration curve for disaccharide ΔDi-6S; m_6 = slope of calibration curve for disaccharide ΔDi-6S; V = volume of Test Solution 1 = 100 mL; W = sample weight, in g; D = dilution factor = 50; and F = molecular weight conversion between ΔDi-6S and ΔDi-di(4,6)S = 1.190.

(**f**) The amount of ΔDi-tri(2,4,6)S in µg/g in the sample is calculated as follows:

$$\frac{P_{2,4,6} - b_6}{m_6} \times \frac{V}{W} \times D \times F$$

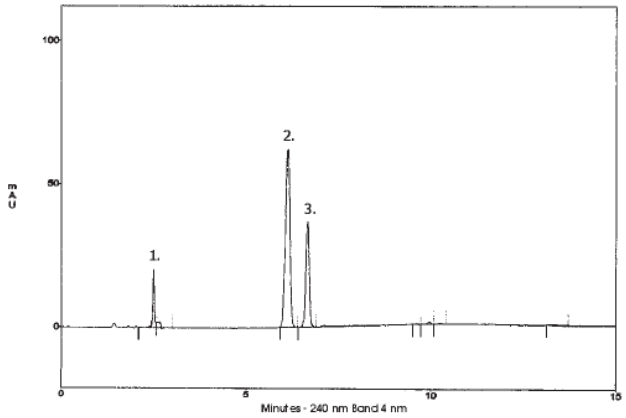

Figure 2015.11B. CS raw material from bovine trachea sample chromatogram. Peak assignments: (*1*) ΔDi-0S, (*2*) ΔDi-6S, and (*3*) ΔDi-4S.

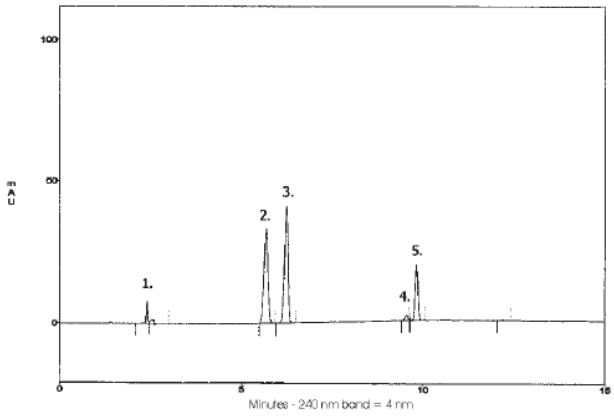

Figure 2015.11C. CS raw material from shark cartilage sample chromatogram. Peak assignments: (*1*) ΔDi-0S, (*2*) ΔDi-6S, (*3*) ΔDi-4S, (*4*) Δdi-di(2,6)S, and (*5*) Δdi-di(4,6)S.

where $P_{2,4,6}$ = peak area of ΔDi-tri(2,4,6)S in sample chromatogram; b_6 = y-intercept of calibration curve for disaccharide ΔDi-6S; m_6 = slope of calibration curve for disaccharide ΔDi-6S; V = volume of Test Solution 1 = 100 mL; W = sample weight, in g; D = dilution factor = 50; and F = molecular weight conversion between ΔDi-6S and ΔDi-tri(2,4,6)S = 1.380.

(**g**) The total amount of CS in µg/g in the sample is the sum of ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDi-di(2,6)S, ΔDi-di(4,6)S, and ΔDi-tri(2,4,6)S.

(**h**) % (w/w) is calculated from µg/g as follows:

$$\%\ (w/w) = \frac{\mu g / g}{10000}$$

(**i**) Milligrams per tablet (mg/tab) is calculated from µg/g as follows:

$$\frac{\mu g / g}{1000} \times TW$$

where TW = the average tablet weight in grams.

(**j**) Milligrams per capsule (mg/cap) is calculated from µg/g as follows:

$$\frac{\mu g / g}{1000} \times FW$$

where FW= the average capsule fill weight in grams.

(**k**) Milligrams per milliliter (mg/mL) is calculated from µg/g for liquid samples as follows:

$$\frac{\mu g / g}{1000} \times SG$$

where SG = the specific gravity of the sample in g/mL.

G. Validation Design

(**a**) *Linearity*.—The five instrument calibration solutions were injected at the beginning of each chromatographic injection sequence, after every 20 sample injections, and at the end of each sequence. A 5-point standard curve was generated for all three analytes, and the slope, y-intercept, correlation coefficient, and relative standard deviation (RSD) of the standard curve were calculated for using the average peak areas at each calibration point on each day.

(**b**) *Repeatability*.—Four replicates of each of the Materials 1–5 (Table **2015.11A**) representing a CS raw material, a hard-shell capsule product containing CS, a tablet product containing CS, a chewable product containing CS, and a liquid product containing CS were prepared on each of 3 days, for a total of 12 replicate preparations of each material. The within-day, between-day, and total repeatability of the total CS content were calculated. The HorRat value (1) for each material was also calculated. In addition, four replicates of each of the Materials 6–9 were prepared on a single day to demonstrate the applicability of the method to these materials. The within-day repeatability was calculated for these materials.

(**c**) *Accuracy*.—(*1*) *CS raw material*.—Heparin, a related glycosaminoglycan (GAG), was used as a negative control. About 200 mg heparin was transferred into ten 100 mL volumetric flasks; 300 mg bovine trachea CS raw material used in the repeatability study (Material 1 in Table **2015.11A**) was added to three of the flasks, 200 mg of the same CS raw material was added to another three of the flasks, 100 mg of the CS raw material was added to another three of the flasks, and the 10th flask was used as a negative control. Each of the spiked negative controls was prepared and analyzed according to the method on 3 separate days.

(*2*) *Spike recovery of dietary supplement finished products*.—A dietary supplement tablet product containing glucosamine HCl and methyl sulfonylmethane (MSM) was used as a negative control for spike recovery study of dietary supplement finished products. The tablets were first ground to a powder and homogenized. About 500 mg of tablet negative control material was transferred into ten 100 mL volumetric flasks. The tablet negative control was then spiked with the bovine trachea CS raw material used in the repeatability study using the same procedure as described for the CS raw material spike recovery study. Each of the spiked negative controls was prepared and analyzed according to the method on 3 separate days.

(**d**) *Ruggedness*.—A Youden ruggedness study was conducted on the bovine trachea raw material, varying the seven factors presented in Table **2015.11E** (2).

(**e**) *Selectivity*.—The selectivity of the method was demonstrated by injecting solutions of non-CS ingredients typically found in CS-containing dietary supplements, including glucosamine, MSM, vitamins, and minerals, into the chromatographic system after treatment with enzyme. In addition, possible contaminants and/or adulterants, such as carrageenan, dermatan sulfate, and heparin, were subjected to the same sample preparation procedure and injected into the chromatographic system. The potential chromatographic interference of hyaluronic acid (HA) was also investigated.

(**f**) *Stability*.—The stabilities of the chondroitinase AC II enzyme in solution and the sample solution were evaluated over the course of the study.

(*1*) *Enzyme stability*.—A portion of the enzyme solution used to prepare the precision samples from Day 1 was stored at –20°C.

Table 2015.11E. Youden ruggedness testing

Parameter	High value	Low value	Factor
Sonication time, min	A = 30	a = 15	0.45
Sample weight, mg CS	B = 200	b = 100	–0.45
Digestion temperature, °C	C = 42	c = 37	–0.8
Concentration of enzyme solution	D = 5 units/0.5 mL	d = 5 units/1.0 mL	–0.05
pH of TRIS buffer solution	E = 7.1	e = 7.5	0.8
Injection volume, µL	F = 50	f = 30	1.3
Detector wavelength, nm	G = 240	g = 235	–0.6

A sample of the bovine trachea CS was prepared and tested after 1 week using this enzyme solution after warming to room temperature. The result from this experiment was compared to the average result obtained in the repeatability study for this material.

(*2*) *Sample solution stability*.—A portion of one of the digested bovine CS sample solutions prepared on Day 1 of the precision study was retained and injected on Day 2 of the precision study. The result from this experiment was compared to the average results obtained in the precision study for this material.

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J. AOAC Int. **90**, 659(2007)

J. AOAC Int. (future issue)

AOAC SMPR 2014.009
J. AOAC Int. **98**, 1058(2014)
DOI: 10.5740/jaoac.int.SMPR2014.009

Posted: August 31, 2015

DIETARY SUPPLEMENTS

Determination of Chondroitin Sulfate Content in Raw Materials and Dietary Supplements by High-Performance Liquid Chromatography with UV Detection After Enzymatic Hydrolysis: Single-Laboratory Validation First Action 2015.11

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A previously validated method for determination of chondroitin sulfate in raw materials and dietary supplements was submitted to the AOAC Expert Review Panel (ERP) for Stakeholder Panel on Dietary Supplements Set 1 Ingredients (Anthocyanins, Chondroitin, and PDE5 Inhibitors) for consideration of First Action *Official Methods*SM status. The ERP evaluated the single-laboratory validation results against AOAC *Standard Method Performance Requirements* 2014.009. With recoveries of 100.8–101.6% in raw materials and 105.4–105.8% in finished products and precision of 0.25–1.8% RSD_r within-day and 1.6–4.72% RSD_r overall, the ERP adopted the method for First Action *Official Methods* status and provided recommendations for achieving Final Action status.

Under a contract from the National Institutes of Health Office of Dietary Supplements, AOAC INTERNATIONAL created the Stakeholder Panel on Dietary Supplements (SPDS), whose mission is to establish voluntary consensus standards for 25 high-priority ingredients. The high-priority ingredients are those for which scientifically valid methods were lacking at the time. As with all stakeholder panels, AOAC engaged industry, government, and academic experts to populate the panel and drive consensus.

In September 2014, SPDS finalized and approved *Standard Method Performance Requirements* (SMPR®) for determination of total chondroitin sulfate (CS) in dietary ingredients and supplements, SMPR 2014.009 (1). This SMPR was intended to outline the minimum recommended performance characteristics for a reference method for routine analysis or dispute resolution. SMPRs are used by AOAC Expert Review Panels (ERPs) as a basis for evaluating candidate methods.

A call for methods was issued by AOAC on January 13, 2015, to select and evaluate methods for CS in dietary supplements according to SMPR 2014.009. The ERP for SPDS Set 1 Ingredients (Anthocyanins, Chondroitin, and PDE5 Inhibitors) considered four methods for CS, and only the method developed by Ji et al. (2) was adopted for *Official Methods* First Action status.

SMPR 2014.009 and LC Method

The LC method and its single-laboratory validation (SLV) were first described in 2007 (2) and in 2015 were submitted to the ERP for SPDS Set 1 Ingredients in response to the call for methods. Briefly, 200 mg of raw material, ground tablets, or ground capsule contents is dissolved in 100 mL water with sonication and filtered if needed. Liquid formulations (200 mg) are diluted to 100 mL with water. The resulting test solution is subjected to hydrolysis with chondroitinase AC II to produce un-, mono-, di-, and trisulfated unsaturated disaccharides. Samples are then analyzed by ion-pairing reverse-phase LC with UV detection, and total CS is determined by summing the amounts of individual disaccharides.

SMPR 2014.009 (1) describes the minimum method performance requirements established by SPDS as summarized in Table 1. In a single-laboratory evaluation, methods must have an LOQ of 1% (w/w); relative SD of repeatability of ≤3% in the low analytical range of 1–10% and ≤2% in the high analytical range of >10–100%; and recovery of 92–105% in the low range and 98–102% in the high range. The matrices to be included in the validation are tablets, capsules, softgels, gel caps, gummies, chewables, liquids, and powders.

The LC method was validated for raw material, capsules, chewables, tablets, softgels, and liquid supplements. Selectivity was evaluated by analyzing CS in the presence of other common dietary supplement ingredients including calcium sulfate, magnesium chloride, zinc chloride, cupric sulfate, glucosamine HCl, methyl sulfonylmethane, chromium(III) chloride, dermatan sulfate, and carrageenan to look for interference with the method, either by deactivating the enzyme or interfering with the LC. Recoveries of CS varied from 97.3 to 102%, demonstrating no interference from the ingredients tested. In addition, hyaluronic acid (HA) was analyzed by the method in the absence of CS and, as expected, produced a signal for ΔDi-0S. HA is hydrolyzed by chondroitinase AC II to generate $\Delta Di\text{-}0S_{HA}$, a diastereomer of ΔDi-0S that cannot be

Received September 18, 2015.
This method was approved by the Expert Review Panel for Dietary Supplements as First Action.
The Expert Review Panel for Dietary Supplements invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
This work was supported by National Institutes of Health Office of Dietary Supplements Contract HHSN263201300015C.
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DOI: 10.5740/jaoacint.15-0220

Table 1. SMPR 2014.009 requirements and Method 2015.11 results

Type of study	Parameter	Minimum acceptable criteria		LC method results
Single-laboratory validation	LOQ	1% (w/w)		NR[a]
	Analytical range	1–10% (w/w)	>10–100% (w/w)	5–100%
	Repeatability (RSD_r)	≤3%	≤2%	0.25–1.8% within-day; 1.60–4.72% total
	Recovery	92–105%	98–102%	100.8–101.6% at 33–60% (w/w) RM[b] 105.4–105.8% at 16.7–37.5% (w/w) FP[c]
Multilaboratory validation	Reproducibility (RSD_R)	≤6%	≤4%	ND[d]

[a] NR = Not reported.

[b] RM = Raw material.

[c] FP = Finished product.

[d] ND = Not determined.

resolved under the LC conditions. Because HA is considerably more expensive than CS, however, it is unlikely to be used as an adulterant.

The linearity of the 5-point calibration curves was demonstrated over a range of 0.2–10 µg/mL ΔDi-0S, 1.4–70 µg/mL ΔDi-4S, and 2–100 µg/mL ΔDi-6S by showing no trend in the residual plots. Values for the coefficient of determination (r) were all >0.999.

Recovery was determined by spiking CS into raw material (heparin was used as a control raw material) and a non-CS commercial tablet product containing glucosamine HCl and methyl sulfonylmethane. Spiked raw material contained 33, 50, and 60% CS by weight, corresponding to 50, 100, and 200% of typical CS amounts in dietary supplements. Spiked finished product contained 16.7, 28.6, and 37.5% CS by weight, corresponding to 50, 100, and 150% of typical CS amounts in dietary supplements. Samples were analyzed in triplicate on 3 days. The method yielded recoveries of 100.8–101.6% over the three levels in raw material and 105.4–105.8% over the three levels in finished product. Repeatability from the spiked samples was 0.98–2.8% RSD_r in raw material and 2.0–3.5% RSD_r in finished product.

Repeatability was determined in three raw materials, two tablets, capsules, chewables, softgels, and liquid supplements by testing four replicate preparations on either 1 or 3 days. Within-day repeatability ranged from 0.25 to 1.8% RSD_r, between-day repeatability ranged from 1.32 to 4.66% RSD_r, and total repeatability ranged from 1.60 to 4.72% RSD_r. Interestingly, the liquid supplement was found not to contain CS, but, when spiked, yielded 99.6% recovery, demonstrating the applicability of the method to liquid supplements.

Finally, a Youden ruggedness trial demonstrated no effect from variation of seven parameters including sample sonication time, sample weight, enzyme hydrolysis time, enzyme concentration, enzyme buffer pH, injection volume, and detector wavelength.

AOAC Official Method 2015.11
Chondroitin Sulfate Content in Raw Materials and Dietary Supplements
High-Performance Liquid Chromatography with UV Detection After Enzymatic Hydrolysis
First Action 2015

Refer to the published method for further details (2).

Discussion

Table 1 provides a comparison of the SMPR and the LC method SLV results. The validation study demonstrated acceptable results for within-day repeatability, although the range of total repeatability (within-day + between-day) exceeded the limit. Recovery was within the SMPR allowable range when spiked surrogate raw material was tested, but recovery from spiked finished product was slightly higher than the allowable range. After careful consideration, the ERP voted on August 3, 2015, to adopt the LC method for First Action *Official Methods* status.

Before obtaining Final Action status, the ERP recommended the following actions: (*1*) optimize and control the moisture in the CS including appropriate vessels and glassware; (*2*) investigate alternate LC columns; (*3*) optimize the LC conditions; (*4*) review lessons learned from the U.S. Pharmacopeia; (*5*) include a potency evaluation of the enzyme used; (*6*) investigate use of the currently available U.S. Pharmacopeia standard; and (*7*) use a certified reference material.

References

(1) AOAC SMPR 2014.009 (2014) *J. AOAC Int.* **98**, 1058–1059

(2) Ji, D., Roman, M., Zhou, J., & Hildreth, J. (2007) *J. AOAC Int.* **90**, 659–669

AOAC AWARDS- **2016** METHOD OF THE YEAR (ERP Chair Nomination Form)

Submission Date	2016-04-25 11:57:55
Name	Brian Schaneberg
Panel Name	SPDS Chondoitin
E-mail	bschaneb@starbucks.com
Date	08-03-2015
AOAC Method Number	2015.11
Method Name	Chondroitin Sulfate Content in Raw Materials and Dietary Supplements High-Performance Liquid Chromatography with Ultraviolet Detection After Enzymatic Hydrolysis
Rationale (based on selection criteria)	The accurate testing of chondroitin in raw materials and finished products has long been a challenge. This method had to go through several modifications/updates in order to achieve the method performance required to meet First Action. One key area was the analysis of the different sources of the enzymes. The method now incorporates an enzyme qualification and activity check in order to be qualified for the method to be considered acceptable. At the end of the day, the development and validation of this method took an extraordinary amount of energy to achieve its First Action status. This will go far in ensuring uniform labeling of chondroitin products and the ability to compare future clinical research.

AOAC Official Method 2015.12
Screening and Identification of Phosphodiesterase Type 5 Inhibitors
in Dietary Ingredients and Supplements
Liquid Chromatography/Quadrupole–Orbital Ion Trap Mass Spectrometry
First Action 2015

(Applicable to screening and identification of acetaminotadalafil, acetildenafil, avanafil, homo sildenafil, hydroxyacetildenafil, hydroxyhomosildenafil, hydroxythiohomosildenafil, lodenafil carbonate, mirodenafil, propoxyphenyl homohydroxysildenafil, sildenafil, tadalafil, thiohomosildenafil, udenafil, vardenafil and other known and novel analogues of the above PDE5 inhibitors.)

A. Apparatus

(a) *LC-MS system.*—UltiMate 3000 LC system (or an equivalent LC system) with Q-Exactive Plus MS (Thermo Fisher Scientific; San Jose, CA, USA) equipped with electrospray ionization [or an equivalent high resolution MS/MS) instrument.
(b) *Analytical balances.*—Accurate to two- and four-decimal places.
(c) *Gilson positive displacements pipets.*—Assorted for 100-1000 µL.
(d) *Repeater pipet.*—For 10 µL - 50-mL size tips.
(e) *Horizontal shaker.*—Shaking speed at least 250 rpm.
(f) *Centrifuge.*—Relative centrifugal force (rcf) at least 3000 *g*.
(g) *Volumetric flasks.*—Class A, glass, assorted sizes.
(h) *Laboratory glassware.*—Class A, various.
(i) *Disposable polypropylene centrifuge tubes.*—15 and 50 mL.
(j) *Disposable plastic syringes.*—3 mL.
(k) *Syringe filters.*—PVDF, 0.22 µm.
(l) *LC vials and caps.*
(m) *Chromatographic column.*—Thermo Fisher Scientific Accucore aQ, 2.6 µm, 100 x 2.1 mm, Part No. 17326-102130.

B. Materials and Reagents

(a) *Methanol (MeOH).*—LC-MS and HPLC grade.
(b) *Water (H_2O).*—LC-MS grade or deionized.
(c) *Acetonitrile (ACN).*—LC-MS and HPLC grade.
(d) *Chloroform.*—HPLC grade.
(e) *Ammonium acetate (NH_4OAc).*—LC-MS grade.
(f) *Formic acid (FA).*—LC-MS grade.

C. Reference Standards

Reference standards (purity ≥ 95%) listed in Table **2015.12A** were purchased from Toronto Research Chemicals (Toronto, ON, Canada), Cachesyn (Mississagua, ON, Canada), TLC Pharmachem (Vaughan, ON, Canada), and Sigma-Aldrich (St. Louis, MO, USA).

D. Preparation of Reagent Solutions and Standards

(a) *50:50 (v/v) ACN:H_2O.*—Combine 500 mL of HPLC grade ACN and 500 mL of deionized H_2O. Sonicate for 2 min.
(b) *70:30 (v/v) H_2O:ACN.*—Combine 700 mL of deionized H_2O and 300 mL of HPLC grade ACN. Sonicate for 2 min.

(c) *LC Mobile Phase A.*—Weigh 0.63 ± 0.01 g of NH_4OAc into appropriate reservoir, add 1000 mL of H_2O and 1 mL of FA. Mix thoroughly.

(d) *LC Mobile Phase B.*—Weigh 0.63 ± 0.01 g of NH_4OAc into appropriate reservoir, add 500 mL of MeOH and sonicate for approx. 3 minutes. Add 500 mL of ACN and 1 mL of FA. Mix thoroughly.

(e) *Individual Stock Solutions.*—Prepare individual solutions of PDE5 inhibitors at concentrations ranging from 1500 to 4000 mg/mL. For aminotadalafil, benzyl sildenafil, chloropretadalafil, desmethylene tadalafil, lodenafil carbonate, tadalafil, and thioaildenafil use a mixture of MeOH and chloroform (2:1, *v/v*). For the remaining analytes use MeOH. If needed sonicate at approx. 30°C to allow for complete dissolution of the solid standard.

(f) *Mixed Stock Standard Solution.*—Combine individual stock solutions of analytes to prepare a composite solution at 20 µg/mL in MeOH.

(g) *Internal Standard (ISTD) Solution.*—Prepare a solution at 20 µg/mL in MeOH using a stock solution of pyrazole N-demethyl sildenafil-d_3.

(h) *QC Solvent Standard.*—Accurately transfer 125 µL of the Mixed Stock Standard Solution and 125 µL of the Internal Standard Solution into a 10-mL volumetric flask. Dilute to volume with 70:30 (*v/v*) H_2O:ACN solution.

E. Safety

See AOAC Official Methods of Analysis (2005), Appendix B: Laboratory Safety. Use appropriate personal protective equipment such as lab coat, safety glasses, rubber gloves, and a fume hood. Dispose of solvents and solutions according to federal, state, and local regulations.

F. Sample Preparation

(a) *Homogenization and Storage of Samples.*—Solid samples such as botanical powders, extracts and tablets are blended to obtain homogenous samples and stored at –4°C. Softgels, gelcaps and capsules are homogenized using cryogenic grinding with liquid nitrogen and stored at –70°C. Liquid samples are briefly shaken and stored at –4°C.

(b) *Extraction Procedure.*

1. Weigh 1.00 ± 0.02 g of thoroughly homogenized sample into a 50-mL centrifuge tube.
2. Add 20 mL of 50:50 (*v/v*) ACN:H_2O solution, briefly hand-shake / vortex and then shake for 15 minutes using a horizontal shaker set at approximately 250 rpm.
3. Centrifuge the tube at >3000 *g* for 5 minutes.
4. Transfer 1 mL of the supernatant to another 50-mL centrifuge tube.
   Note: When transferring extract aliquots obtained for softgels, avoid the upper lipophilic layer formed during the centrifugation step.
5. Add 19 mL of 70:30 (*v/v*) H_2O:ACN solution and vortex mix briefly.
6. Filter approximately 3-mL of the diluted extract using plastic syringe fitted with a 0.22 µm PVDF syringe filter into a 15-mL centrifuge tube.
7. Transfer 1 mL of the filtrate to a 2-mL autosampler vial and add 12.5 µL of the Internal Standard Solution.
8. Cap the vial and vortex mix briefly.
9. Perform LC-HRMS analysis.

G. LC-HRMS Analysis

(a) *LC Operating Conditions.*

1. Column: Thermo Scientific Accucore aQ, 2.6 µm, 100 x 2.1 mm

2. Column temperature: 30°C
3. Mobile phase A: 10 mM NH_4OAc and 0.1% FA in H_2O
4. Mobile phase B: 10 mM NH_4OAc and 0.1% FA in ACN-MeOH (50:50, *v/v*)
5. Flow rate: 0.3 mL/min
6. Elution gradient see Table **2015.12B**
7. Injection volume: 3 µL
8. Autosampler temp.: 15°C
9. Run time: 25 min

(b) *MS Data Acquisition and Operating Conditions.*—MS data acquisition is performed in full MS-data dependent product ion scan (dd-MS^2) and all ion fragmentation (AIF) modes using parameter settings provided below. Data dependent product ion scan experiment is initiated if a mass (*m/z*) specified in an inclusion list (see Table **2015.12B**) is detected in correct retention time window within a mass error of 10 ppm and at the intensity above the set threshold level. The ion fragmentation in AIF and dd-MS^2 modes is performed at three discrete normalized collision energy (NCE) values.

1. Ionization mode: positive ESI
2. Sheath gas flow: 35 arb
3. Aux gas flow: 10 arb
4. Sweep gas flow: 1 arb
5. Spray voltage: 3.5 kV
6. Capillary temperature: 350°C
7. S-lens RF level: 50 V
8. Aux gas heater temp.: 350°C
9. Full MS resolution: 70,000 FWHM
10. Full MS AGC target: 1e6
11. Full MS max IT: 100 ms
12. Full MS scan range: *m/z* 200-1100
13. dd-MS2 resolution: 17,500 FWHM
14. dd-MS2 AGC target: 1e5
15. dd-MS2 Isolation window: 1.0 Da
16. dd-MS2 stepped NCE: 40, 70, 100%
17. Intensity threshold: 2.0e4
18. AIF resolution: 70,000 FWHM
19. AIF AGC target: 1e6
20. AIF max IT: 100 ms
21. AIF stepped NCE: 40, 70, 100%
22. AIF scan range: *m/z* 50-750

(c) Inclusion list .—See Table **2015.12C**.

(d) *Positive and Negative Control.*—Analyze a reagent blank (a *negative control*) with each sample set. Inject the QC Solvent Standard (a *positive control*) at the beginning, after every ten samples and at the end of the LC-HRMS sequence. The ISTD response in samples should be within 40-140% of its average response in the QC Solvent Standards.

H. Data Processing

(a) *Workflow and Detection / Identification Criteria.*—Detection and identification of analytes is performed with the use of TraceFinder software and settings provided below. Detection of targeted PDE5 inhibitors is based on automatic comparison of retention times of peaks extracted from full MS record and accurate mass of respective pseudomolecular ion $[M+H]^+$ with information in TraceFinder compound database (see Table **2015.12D**). Retention time and mass tolerances of 30 s and 5 ppm, respectively, are used. To identify an analyte, additional criteria have to be fulfilled. These include mass accuracy (Δ *m/z* ≤ 5 ppm) and relative responses (10% tolerance) of pseudomolecular ion isotopes, as well as criteria for fragment ions detected in appropriate dd-MS^2 records. For positive identification, one or more fragment ions listed in the TraceFinder compound database have to be detected above the intensity threshold with a mass error ≤ 5 ppm. The detection / identification workflow for targeted compounds is provided in Figure **2015.12**. PDE5 inhibitors not included in the TraceFinder compound database can be detected and identified by extraction of respective pseudomolecular ions from full MS records and evaluation of fragment ions in AIF records. A search using common PDE5 inhibitor fragments can be utilized to highlight components with structures similar to known PDE5 inhibitors.

(b) TraceFinder Software Settings.

1.	Retention time range:	1-23 min
2.	Peak area threshold:	100,000
3.	Signal-to-noise threshold:	10
4.	Mass tolerance (parent ion):	5 ppm
5.	RT tolerance:	30 s
6.	Minimum # of fragments:	1
7.	Intensity threshold:	1,000
8.	Mass tolerance (fragment ion):	5 ppm
9.	Isotope pattern fit threshold:	95%
10.	Mass tolerance (isotope):	5 ppm
11.	Intensity tolerance (isotope):	10%

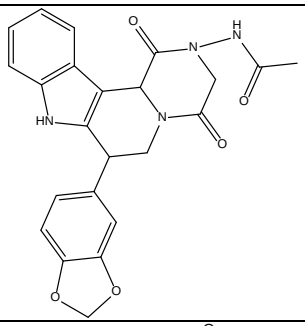
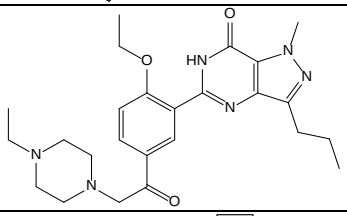
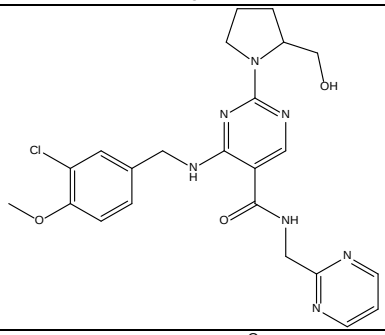
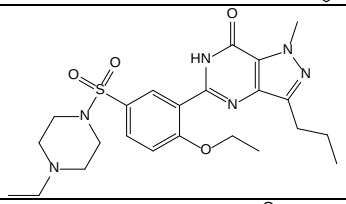
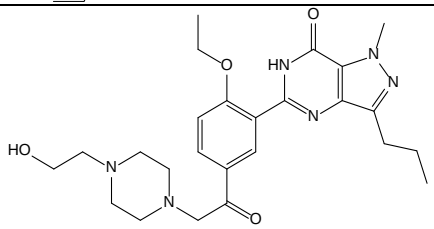
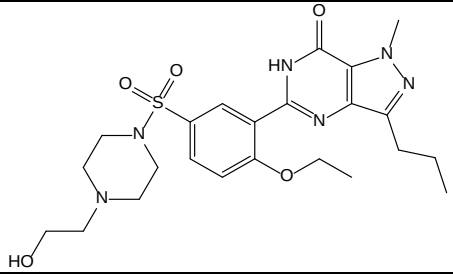
(c) *TraceFinder Compound Database.*–Compound database (see Table **2015.12D**) comprises information on the exact mass of pseudomolecular ions, molecular formulas, retention times and exact masses of 8-10 fragment ions for each analyte. The *m/z* values of fragments in the compound database represent exact masses that were calculated using experimental data obtained by HRMS analysis of reference standards and elucidation of fragment ions in Mass Frontier spectral interpretation software or based on information available in m/zCloud database and scientific literature.

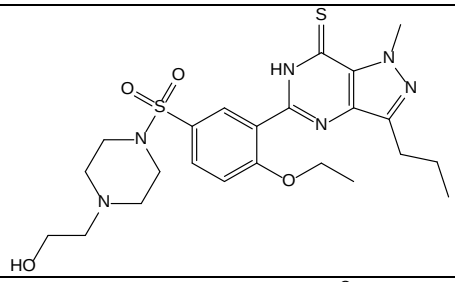
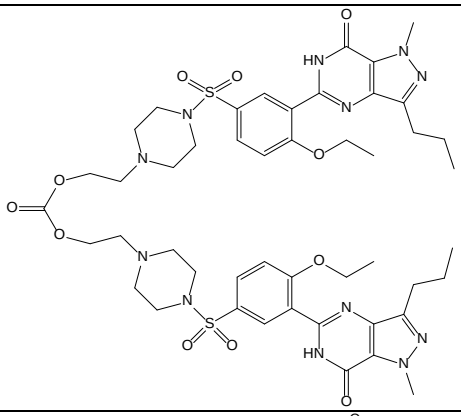
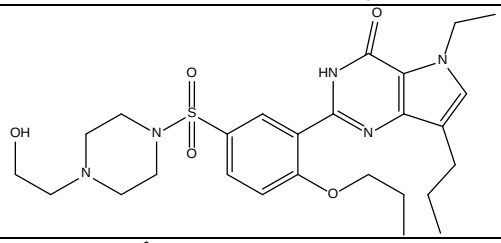
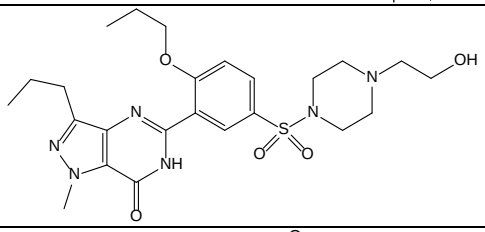
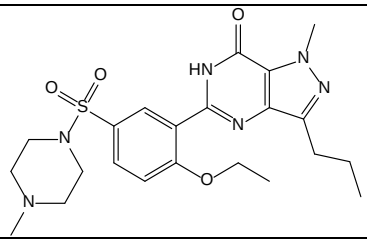
Reference: *J. AOAC Int*. (future issue)

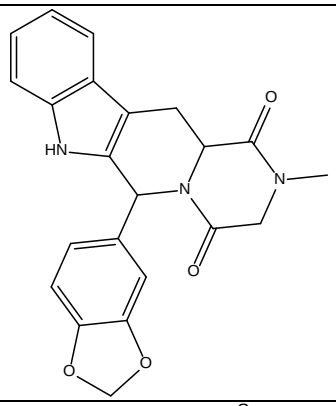
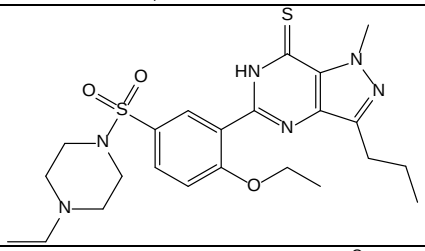
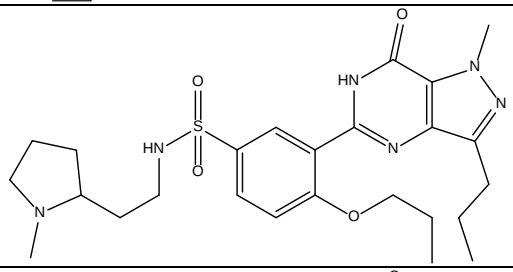
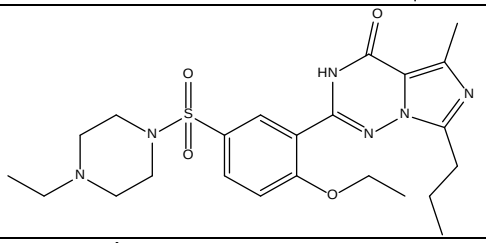
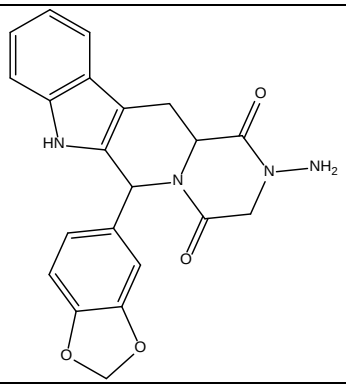
AOAC SMPR 2014.010
J. AOAC Int. **98**, 1060(2015)

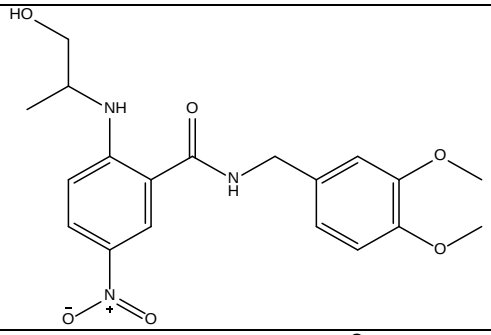
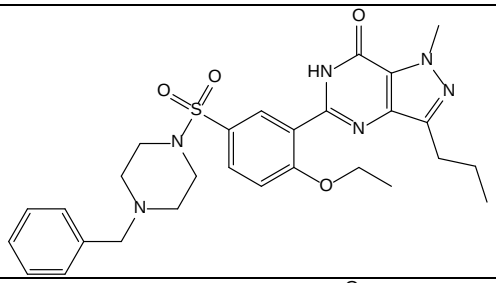
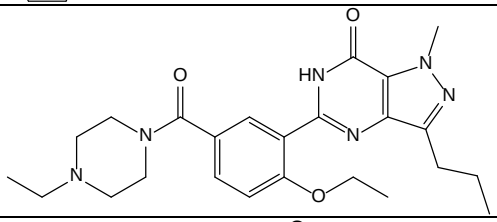
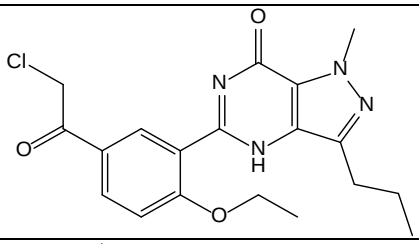
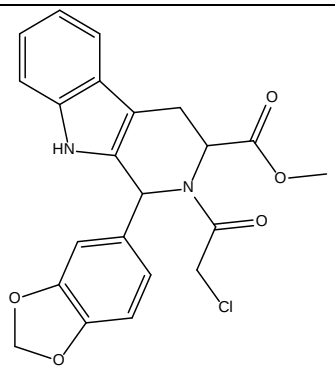
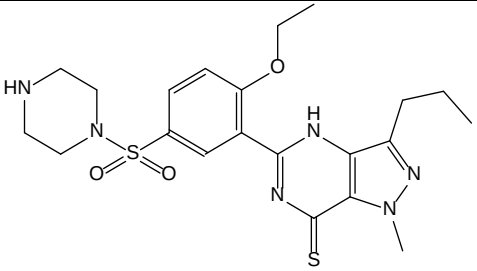
Posted: August 2015

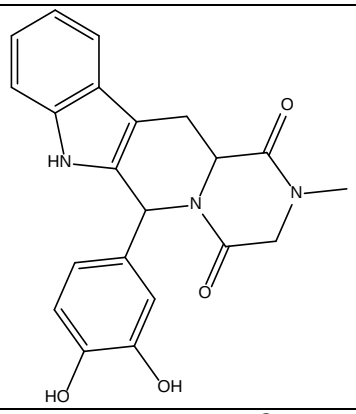
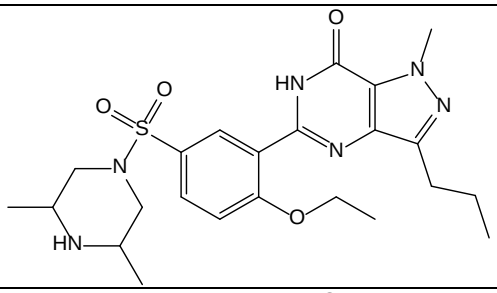
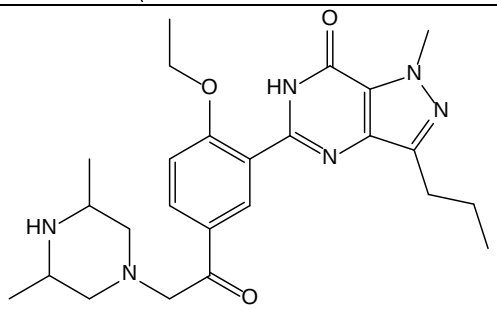
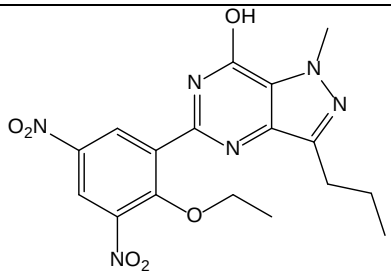
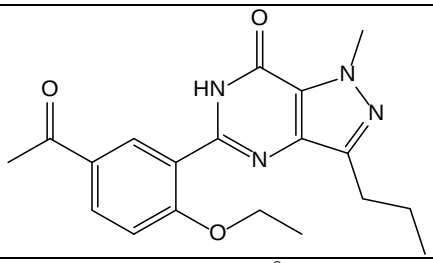
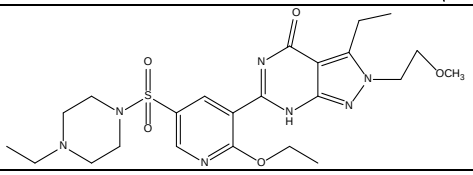
Table 2015.12A. Overview of PDE5 inhibitors analyzed in the study

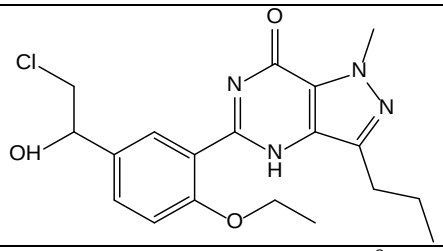
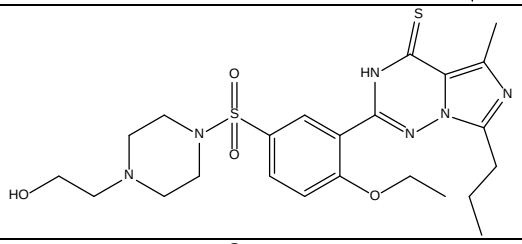
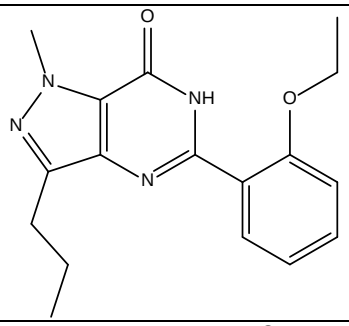
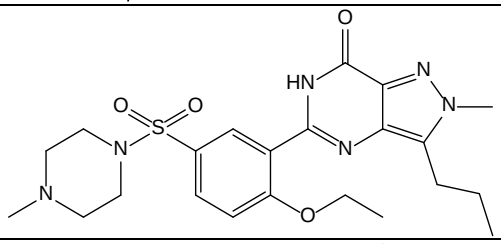
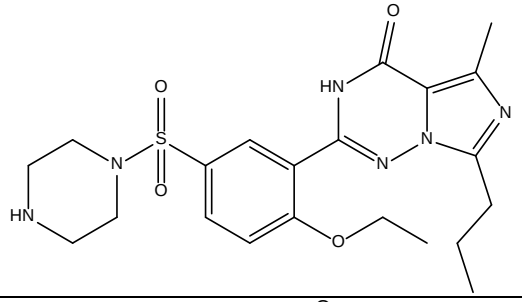
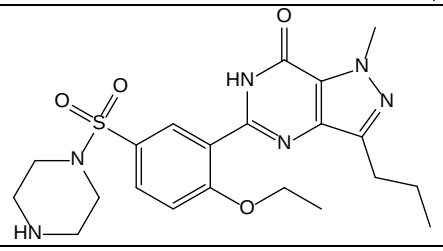
Analyte	CAS No.	Formula	Note	Structure
Acetaminotadalafil	1446144-71-3	$C_{23}H_{20}N_4O_5$	Target panel	
Acetildenafil	831217-01-7	$C_{25}H_{34}N_6O_3$	Target panel	
Avanafil	330784-47-9	$C_{23}H_{26}ClN_7O_3$	Target panel	
Homosildenafil	642928-07-2	$C_{23}H_{32}N_6O_4S$	Target panel	
Hydroxyacetildenafil	147676-56-0	$C_{25}H_{34}N_6O_4$	Target panel	
Hydroxyhomosildenafil	139755-85-4	$C_{23}H_{32}N_6O_5S$	Target panel	

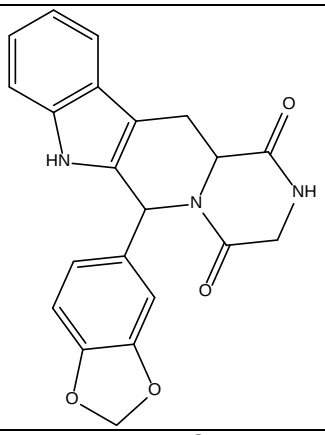
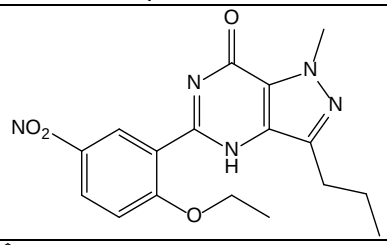
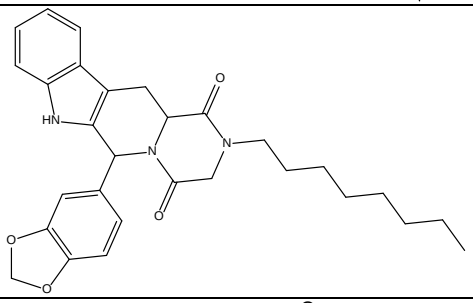
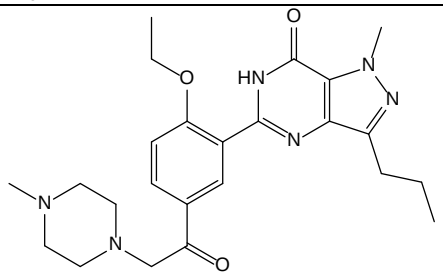
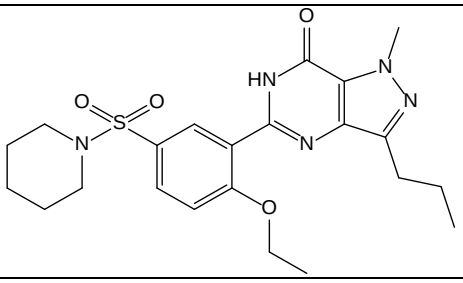
Analyte	CAS No.	Formula	Note	Structure
Hydroxythiohomo sildenafil	Not available	$C_{23}H_{32}N_6O_4S_2$	Target panel	
Lodenafil carbonate	398507-55-6	$C_{47}H_{62}N_{12}O_{11}S_2$	Target panel	
Mirodenafil	862189-95-5	$C_{26}H_{37}N_5O_5S$	Target panel	
Propoxyphenyl homohydroxysildenafil	139755-87-6	$C_{24}H_{34}N_6O_5S$	Target panel	
Sildenafil	139755-83-2	$C_{22}H_{30}N_6O_4S$	Target panel	

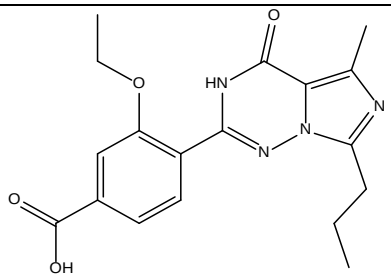
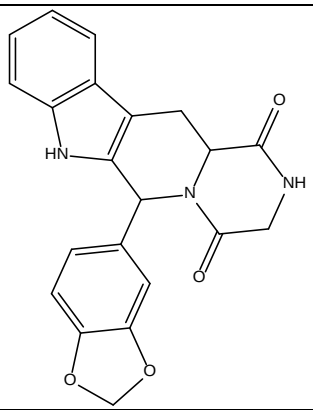
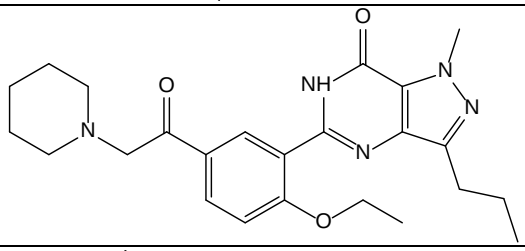
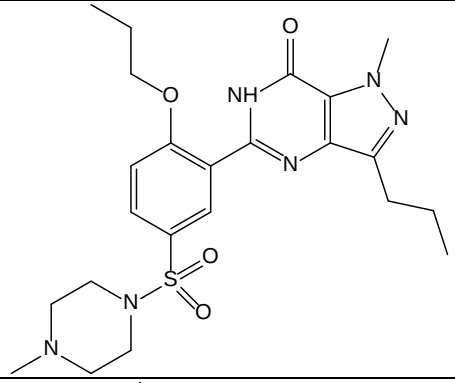
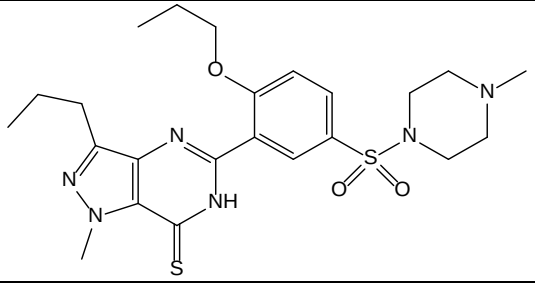
Analyte	CAS No.	Formula	Note	Structure
Tadalafil	171596-29-5	$C_{22}H_{19}N_3O_4$	Target panel	
Thiohomosildenafil	479073-80-8	$C_{23}H_{32}N_6O_3S_2$	Target panel	
Udenafil	268203-93-6	$C_{25}H_{36}N_6O_4S$	Target panel	
Vardenafil	224789-15-5	$C_{23}H_{32}N_6O_4S$	Target panel	
Aminotadalafil	385769-84-6	$C_{21}H_{18}N_4O_4$	-	

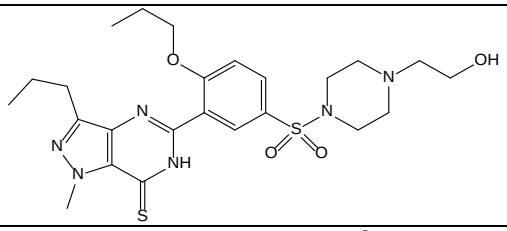
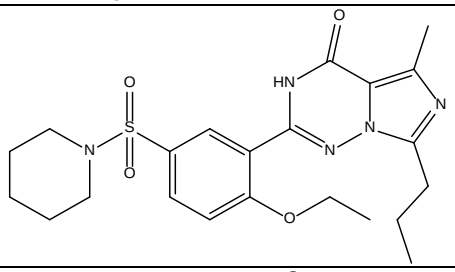
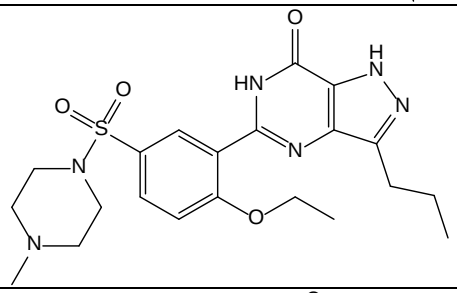
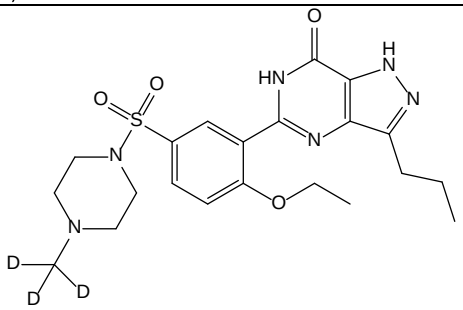
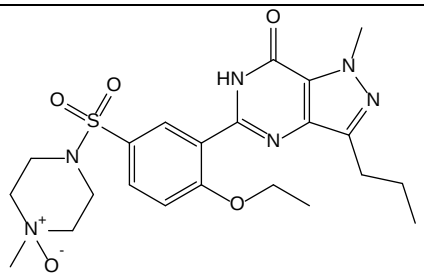
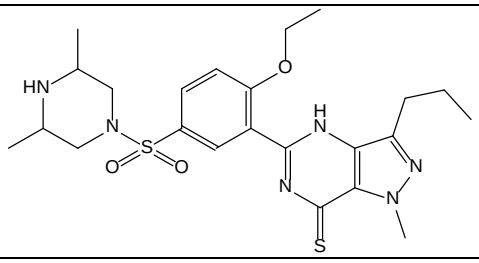
Analyte	CAS No.	Formula	Note	Structure
Benzamidenafil	1020251-53-9	$C_{19}H_{23}N_3O_6$	-	
Benzylsildenafil	Not available	$C_{28}H_{34}N_6O_4S$	-	
Carbodenafil	Not available	$C_{24}H_{32}N_6O_3$	-	
Chlorodenafil	1058653-74-9	$C_{19}H_{21}ClN_4O_3$	-	
Chloropretadalafil	171489-59-1	$C_{22}H_{19}ClN_2O_5$	-	
Desmethylthiosildenafil	479073-86-4	$C_{21}H_{28}N_6O_3S_2$	-	

Analyte	CAS No.	Formula	Note	Structure
Desmethylenetadalafil	171489-03-5	$C_{21}H_{19}N_3O_4$	-	
Dimethylsildenafil	496835-35-9	$C_{23}H_{32}N_6O_4S$	-	
Dimethylacetildenafil	Not available	$C_{25}H_{34}N_6O_3$	-	
Dinitrodenafil	Not available	$C_{17}H_{18}N_6O_6$	-	
Gendenafil	147676-66-2	$C_{19}H_{22}N_4O_3$	-	
Gisadenafil	334827-98-4	$C_{23}H_{33}N_7O_5S$	-	

Analyte	CAS No.	Formula	Note	Structure
Hydroxychlorodenafil	1391054-00-4	$C_{19}H_{23}ClN_4O_3$	-	
Hydroxythiovardenafil	912576-30-8	$C_{23}H_{32}N_6O_4S_2$	-	
Imidazosagatriazinone	139756-21-1	$C_{17}H_{20}N_4O_2$	-	
Isosildenafil	253178-46-0	$C_{22}H_{30}N_6O_4S$	-	
N-Desethyl vardenafil	448184-46-1	$C_{21}H_{28}N_6O_4S$	-	
N-Desmethyl sildenafil	139755-82-1	$C_{21}H_{28}N_6O_4S$	-	

Analyte	CAS No.	Formula	Note	Structure
N-Desmethyl tadalafil	171596-36-4	$C_{21}H_{17}N_3O_4$	-	
Nitrodenafil	147676-99-1	$C_{17}H_{19}N_5O_4$	-	
N-Octyl nortadalafil	1173706-35-8	$C_{29}H_{33}N_3O_4$	-	
Noracetildenafil	949091-38-7	$C_{24}H_{32}N_6O_3$	-	
Norneosildenafil	371959-09-0	$C_{22}H_{29}N_5O_4S$	-	

Analyte	CAS No.	Formula	Note	Structure
Norneovardenafil	358390-39-3	$C_{18}H_{20}N_4O_4$	-	
Nortadalafil	Not available	$C_{21}H_{17}N_3O_4$	-	
Piperiacetildenafil	Not available	$C_{24}H_{31}N_5O_3$	-	
Propoxyphenyl sildenafil	Not available	$C_{23}H_{32}N_6O_4S$	-	
Propoxyphenyl thiosildenafil	479073-87-5	$C_{23}H_{32}N_6O_3S_2$	-	

Analyte	CAS No.	Formula	Note	Structure
Propoxyphenyl thiohydroxyhomosildenafil	479073-90-0	$C_{24}H_{34}N_6O_4S_2$	-	
Pseudovardenafil	224788-34-5	$C_{22}H_{29}N_5O_4S$	-	
Pyrazole N-demethyl sildenafil	139755-95-6	$C_{21}H_{28}N_6O_4S$	-	
Pyrazole N-demethyl sildenafil-d_3	Not available	$C_{21}H_{25}D_3N_6O_4S$	ISTD	
Sildenafil N-oxide	1094598-75-0	$C_{22}H_{30}N_6O_5S$	-	
Thioaildenafil	856190-47-1	$C_{23}H_{32}N_6O_3S_2$	-	

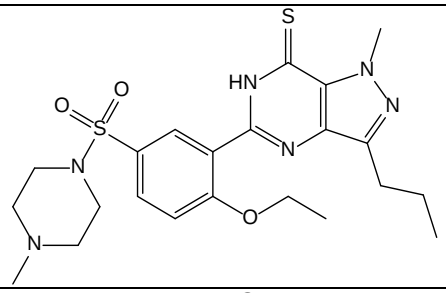
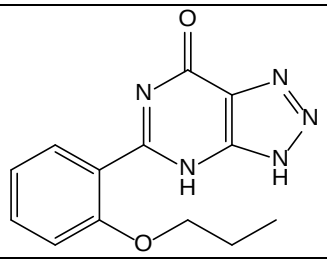
Analyte	CAS No.	Formula	Note	Structure
Thiosildenafil	479073-79-5	$C_{22}H_{30}N_6O_3S_2$	-	
Zaprinast	37762-06-4	$C_{13}H_{13}N_5O_2$	-	

Table 2015.12B. Gradient elution program

Time (min)	A (%)	B (%)
0.00	98	2
0.50	98	2
2.00	60	40
20.00	5	95
23.00	5	95
23.01	98	2
24.00	98	2

Table 2015.12C. Inclusion list used in the dd-MS^2 experiment for the target compound panel

Mass (*m*/z)	Formula	Species	Charge State	Polarity	Start (min)	End (min)
483.27143	C25H34N6O4	+ H	1	Positive	4.06	4.36
467.27652	C25H34N6O3	+ H	1	Positive	4.35	4.65
489.22785	C23H32N6O4S	+ H	1	Positive	4.72	5.02
505.22277	C23H32N6O5S	+ H	1	Positive	4.86	5.16
484.18584	C23H26ClN7O3	+ H	1	Positive	4.88	5.18
475.21220	C22H30N6O4S	+ H	1	Positive	4.92	5.22
489.22785	C23H32N6O4S	+ H	1	Positive	5.08	5.38
433.15065	C23H20N4O5	+ H	1	Positive	5.18	5.48
517.25915	C25H36N6O4S	+ H	1	Positive	5.73	6.03
519.23842	C24H34N6O5S	+ H	1	Positive	5.80	6.10
390.14483	C22H19N3O4	+ H	1	Positive	5.97	6.27
532.25882	C26H37N5O5S	+ H	1	Positive	7.78	8.08
521.19992	C23H32N6O4S2	+ H	1	Positive	8.60	8.90
505.20501	C23H32N6O3S2	+ H	1	Positive	8.92	9.22
1035.41752	C47H62N12O11S2	+ H	1	Positive	12.78	13.08

Table 2015.12D. TraceFinder software compound database for the target compound panel

Compound name	Chemical Formula	Extracted mass	Adduct	RT	Fragment ions (*m/z*)
Acetaminotadalafil	$C_{23}H_{20}N_4O_5$	433.15065	M+H	5.33	204.08078; 262.08626; 135.04406; 205.08860; 233.08352; 232.07569; 169.07602; 191.07295; 263.09408; 250.08626
Acetildenafil	$C_{25}H_{34}N_6O_3$	467.27652	M+H	4.50	111.09167; 97.07602; 70.06513; 84.08078; 72.08078; 127.12297; 112.09950; 297.13460; 56.04948; 166.09749
Avanafil	$C_{23}H_{26}ClN_7O_3$	484.18584	M+H	5.03	155.02582; 375.12184; 105.03349; 77.03858; 95.04914; 53.03858; 357.11128; 233.10330; 67.05423; 221.10330
Homosildenafil	$C_{23}H_{32}N_6O_4S$	489.22785	M+H	5.23	72.08078; 58.06513; 99.09167; 113.10732; 70.06513; 283.11895; 84.08078; 71.07295; 114.11515; 311.15025
Hydroxyacetildenafil	$C_{25}H_{34}N_6O_4$	483.27143	M+H	4.21	97.07602; 70.06513; 127.08659; 143.11789; 100.07569; 297.13460; 88.07569; 166.09749; 112.09950; 128.09441
Hydroxyhomo sildenafil	$C_{23}H_{32}N_6O_5S$	505.22277	M+H	5.01	99.09167; 70.06513; 58.06513; 84.06820; 97.07602; 283.11895; 88.07569; 129.10224; 112.0995; 311.15025
Hydroxythiohomo sildenafil	$C_{23}H_{32}N_6O_4S_2$	521.19992	M+H	8.75	99.09167; 70.06513; 58.06513; 84.06820; 299.09611; 129.10224; 97.07602; 88.07569; 327.12741; 112.09950
Lodenafil carbonate	$C_{47}H_{62}N_{12}O_{11}S_2$	1035.41752	M+H	12.93	112.09950; 82.06513; 97.07602; 111.09167; 487.21220; 83.06037; 84.08078; 283.11895
Mirodenafil	$C_{26}H_{37}N_5O_5S$	532.25882	M+H	7.93	99.09167; 296.13935; 312.13427; 70.06513; 56.04948;84.06820; 210.06619; 129.10224; 88.07569; 121.03964

Compound name	Chemical Formula	Extracted mass	Adduct	RT	Fragment ions (*m/z*)
Propoxyphenyl homohydroxysildenafil	$C_{24}H_{34}N_6O_5S$	519.23842	M+H	5.95	99.09167; 70.06513; 283.11895; 84.06820; 97.07602; 299.11387; 129.10224; 88.07569; 112.09950; 255.12404
Sildenafil	$C_{22}H_{30}N_6O_4S$	475.2122	M+H	5.07	58.06513; 100.09950; 99.09167; 56.04948; 283.11895; 70.06513; 311.15025; 225.07709; 299.11387
Tadalafil	$C_{22}H_{19}N_3O_4$	390.14483	M+H	6.12	204.08078; 135.04406; 262.08626; 169.07602; 205.08860; 232.07569; 233.08352; 240.11314; 268.10805; 250.08626
Thiohomosildenafil	$C_{23}H_{32}N_6O_3S_2$	505.20501	M+H	9.07	72.08078; 99.09167; 113.10732; 56.04948; 299.09611; 70.06513; 84.08078; 327.12741; 71.07295; 355.15806
Udenafil	$C_{25}H_{36}N_6O_4S$	517.25915	M+H	5.88	84.08078; 112.11208; 283.11895; 58.06513; 325.16590; 299.11387; 81.06988; 255.124037; 79.05423; 82.06513
Vardenafil	$C_{23}H_{32}N_6O_4S$	489.22785	M+H	4.87	169.09715; 344.14791; 110.06004; 299.11387; 72.08078; 123.09167; 70.06513; 376.10740; 68.01309; 113.10732

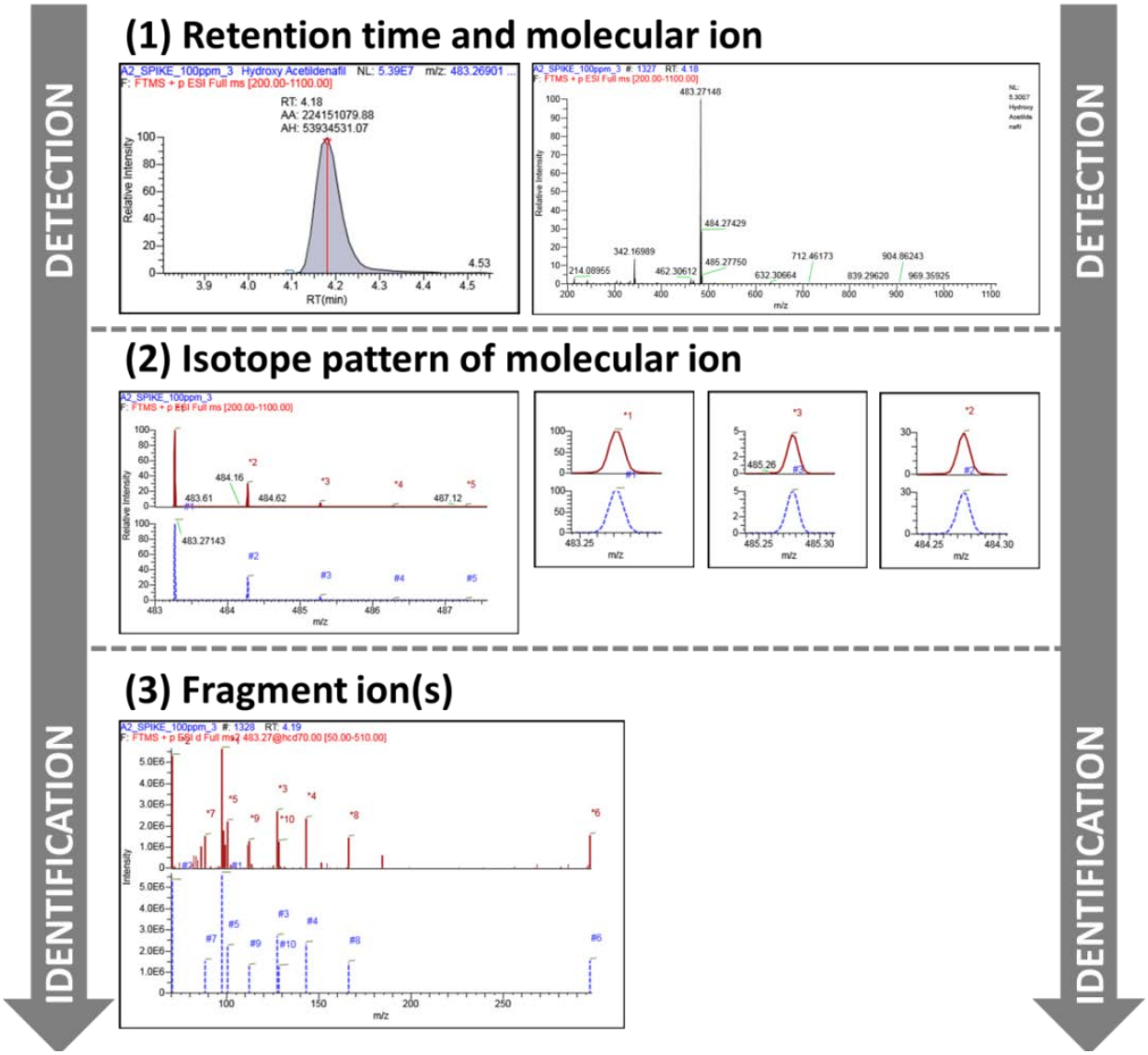


Figure 2015.12. Detection/identification workflow for targeted analytes.

DIETARY SUPPLEMENT

Single-Laboratory Validation Study of a Method for Screening and Identification of Phosphodiesterase Type 5 Inhibitors in Dietary Ingredients and Supplements Using Liquid Chromatography/Quadrupole–Orbital Ion Trap Mass Spectrometry: First Action 2015.12

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A single-laboratory validation study of a method for screening and identification of phosphodiesterase type 5 (PDE5) inhibitors in dietary ingredients and supplements is described. PDE5 inhibitors were extracted from the samples using a 50:50 (v/v) mixture of acetonitrile and water and centrifuged. Supernatant was diluted, filtered, and analyzed by LC–high-resolution MS. Data were collected in MS acquisition mode that combined full-scan MS experiment with all-ion fragmentation and data-dependent MS/MS product from the ion scan experiment. This approach enabled collection of MS and tandem MS (MS/MS) data for both targeted and nontargeted PDE5 inhibitors in a single chromatographic run. Software-facilitated identification of targeted analytes was performed based on the retention time, accurate mass, and isotopic pattern of pseudomolecular ions, and accurate masses of fragment ions using an in-house compound database. Detection and identification of other PDE5 inhibitors and novel analogs were performed by retrospective evaluation of MS and MS/MS experimental data. The method validation results obtained for evaluated matrixes fulfilled the probability of identification requirements and probability of detection requirements (for the pooled data) set at 90% (95% confidence interval) in the respective AOAC *Standard Method Performance Requirements* for identification and screening methods for PDE5 inhibitors. Limited data demonstrating the quantification capability of the method were also generated. Mean recovery and repeatability obtained for the evaluated PDE5 inhibitors were in the range 69–90% and 0.4–1.8%, respectively.

Deliberate addition of active pharmaceutical ingredients to dietary supplements is a profit-driven practice that aims to develop or intensify the claimed biological effect of the product (1, 2). Phosphodiesterase type 5 (PDE5) inhibitors, such as avanafil, lodenafil carbonate, mirodenafil, sildenafil, tadalafil, udenafil, or vardenafil and their unapproved designer analogs, represent an important class of pharmaceuticals that are frequently used to adulterate products advertised to provide an enhancement to sexual performance and ingredients used in their manufacturing (3, 4). Considering that PDE5 inhibitors can negatively interact with certain prescription drugs and that limited knowledge is available on safety and efficacy of the designer analogs, the presence of such compounds in dietary supplements may represent a serious health risk to consumers (2). Therefore, reliable analytical methods are needed for detection, identification, and quantification of PDE5 inhibitors in relevant dietary supplement raw materials and finished products.

To address this problem, AOAC INTERNATIONAL issued a call for methods for screening, identification, and determination of PDE5 inhibitors in dietary ingredients and supplements based on *Standard Method Performance Requirements* (SMPRs®) developed by a working group of the AOAC INTERNATIONAL Stakeholder Panel on Dietary Supplements (5–7). Single-laboratory validation (SLV) requirements provided in AOAC SMPR 2014.010 for identification of PDE5 inhibitors are summarized in Table 1.

Received August 27, 2015.
This method was approved by the Expert Review Panel for Dietary Supplements as First Action.
The Expert Review Panel for Dietary Supplements invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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DOI: 10.5740/jaoacint.15-0202

Table 1. Method performance requirements (AOAC SMPR 2014.010)

Type of study	Study	Parameter	Parameter requirements	Target test concn	Minimum acceptable results
SLV	Matrix study	POI at low concn	Minimum of 33 replicates representing all target compounds in Annex I and ideally all matrix types listed in Annex II, spiked at or below the designated low level target test concentration	100 ppm	90% POI[a] of the pooled data for all target compounds and matrixes
		POI at high concn	Minimum of 5 replicates per matrix type spiked at 10× the designated low level target test concentration	10× low concn	100% correct analyses are expected[b]
		POI at 0 concn	Minimum of 5 replicates per matrix type	0 ppm	

[a] 95% Confidence interval.

[b] 100% Correct analyses are expected. Some aberrations may be acceptable if the aberrations are investigated, and acceptable explanations can be determined and communicated to method users.

SLV Study

This validation study evaluated probability of identification (POI) for 15 target panel PDE5 inhibitors provided in the AOAC SMPR 2014.010 (*see* Table 2). The evaluation was performed at concentrations of 0, 100, and 1000 mg/kg. Considering the availability and cost of the reference standards and amounts needed to obtain the above target concentrations in the samples, postextraction spiking of blank matrix extracts with target panel compounds was performed at 250 and 2500 ng/mL to obtain concentrations corresponding to 100 and 1000 mg/kg in the samples, respectively. Five samples were prepared for each concentration level in each of the seven evaluated matrixes. This experimental design resulted in 35 samples per concentration level and a final set of 105 samples, which fulfilled requirements provided in AOAC SMPR 2014.010. The samples were analyzed using LC–high-resolution MS (LC-HRMS) with a Q-Exactive Plus instrument (Thermo Fisher Scientific, San Jose, CA), followed by raw data processing with TraceFinder software (Thermo Fisher Scientific, San Jose, CA) that allowed for the automatic identification of the target PDE5 inhibitors using the identification criteria discussed below.

To demonstrate the ability of the method to extract PDE5 inhibitors from the samples, a homogenized capsule dietary supplement (M5 in Table 3) was spiked in triplicate with the target panel compounds at 50 mg/kg and extracted according to the method sample preparation protocol. Analyte recoveries were calculated using matrix-matched standards.

The evaluated matrixes covered the dietary ingredient and supplement matrix types provided in Annex II of AOAC SMPR 2014.010: tablets, capsules (both content and capsule shells), softgels, liquid drink, herbal tincture, botanical powder, and botanical extract. Representative samples of each matrix type were selected to cover the variety of typical ingredients used in the manufacture of sexual enhancement supplements. Table 3 lists the samples and ingredients declared by the vendor on the label of the respective product.

AOAC Official Method 2015.12
Screening and Identification of Phosphodiesterase Type 5 Inhibitors in Dietary Ingredients and Supplements Using Liquid Chromatography/ Quadrupole–Orbital Ion Trap Mass Spectrometry
First Action 2015

[Applicable to the screening and identification of acetaminotadalafil, acetildenafil, avanafil, homosildenafil, hydroxyacetildenafil, hydroxyhomosildenafil, hydroxythiohomosildenafil, lodenafil carbonate, mirodenafil, propoxyphenyl homohydroxysildenafil, sildenafil, tadalafil, thiohomosildenafil, udenafil, vardenafil, and other known and novel analogs of the above PDE5 inhibitors.]

Caution: See AOAC Official Methods of Analysis℠ Appendix B: Laboratory Safety (8). Use appropriate personal protective equipment such as a laboratory coat, safety glasses, rubber gloves, and a fume hood. Dispose of solvents and solutions according to federal, state, and local regulations.

A. Apparatus

(a) *LC-MS system.*—UltiMate 3000 LC system (Thermo Fisher Scientific, San Jose, CA) (or an equivalent LC system) with Q-Exactive Plus mass spectrometer equipped with electrospray ionization [or equivalent high-resolution tandem MS (MS/MS)] instrument.

(b) *Analytical balances.*—Accurate to two and four decimal places.

(c) *Gilson positive displacements pipets*.—Assorted for 100–1000 µL.

(d) *Repeater pipet.*—For 10 µL to 50 mL size tips.

(e) *Horizontal shaker.*—Shaking speed at least 250 rpm.

(f) *Centrifuge.*—Relative centrifugal force of at least 3000 × *g*.

(g) *Volumetric flasks.*—Class A, glass, assorted sizes.

(h) *Laboratory glassware.*—Class A, various.

(i) *Disposable polypropylene centrifuge tubes*.—15 and 50 mL.

(j) *Disposable plastic syringes*.—3 mL.

(k) *Syringe filters.*—PTFE, 0.22 µm.

(l) *LC vials and caps*.

(m) *Chromatographic column.*—Thermo Fisher Scientific Accucore aQ C18 (Part No. 17326-102130), 2.6 µm, 100 × 2.1 mm.

(n) *Guard column.*—Thermo Fisher Scientific Accucore aQ C18 (Part No. 17326-012105), 2.6 µm, 10 × 2.1 mm.

B. Materials and Reagents

(a) *Methanol (MeOH).*—LC-MS and HPLC grade.

(b) *Water (H_2O).*—LC-MS grade or deionized.

(c) *Acetonitrile (ACN).*—LC-MS and HPLC grade.

(d) *Chloroform.*—HPLC grade.

(e) *Ammonium formate (NH_4OFor).*—LC-MS grade.

(f) *Formic acid (FA).*—LC-MS grade.

C. Reference Standards

The reference standards (purity ≥95%) listed in Table 2 were purchased from Toronto Research Chemicals (Toronto,

Table 2. Overview of PDE5 inhibitors analyzed in the study

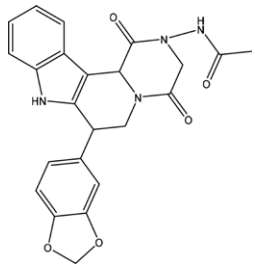
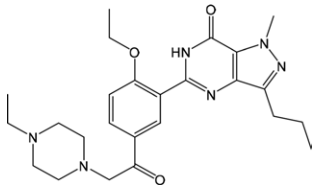
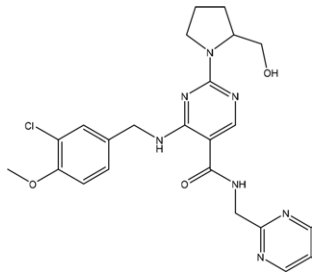
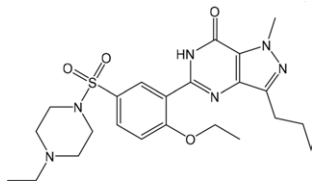
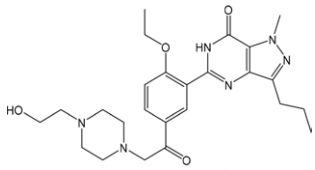
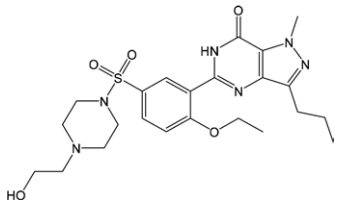
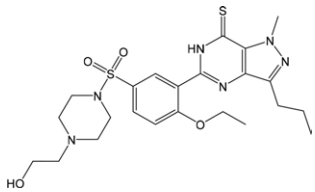
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Acetaminotadalafil	1446144-71-3	$C_{23}H_{20}N_4O_5$	Target panel	
Acetildenafil	831217-01-7	$C_{25}H_{34}N_6O_3$	Target panel	
Avanafil	330784-47-9	$C_{23}H_{26}ClN_7O_3$	Target panel	
Homosildenafil	642928-07-2	$C_{23}H_{32}N_6O_4S$	Target panel	
Hydroxyacetildenafil	147676-56-0	$C_{25}H_{34}N_6O_4$	Target panel	
Hydroxyhomosildenafil	139755-85-4	$C_{23}H_{32}N_6O_5S$	Target panel	
Hydroxythiohomo sildenafil	479073-82-0	$C_{23}H_{32}N_6O_4S_2$	Target panel	

Table 2. (*continued*)

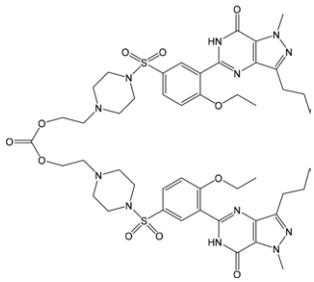
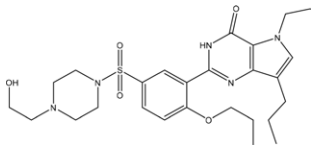
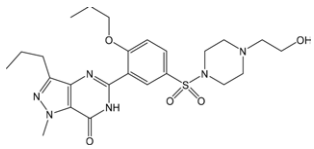
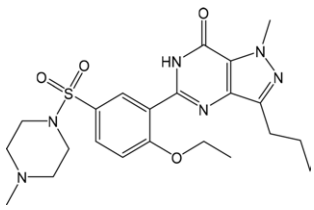
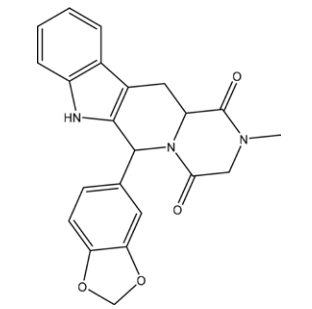
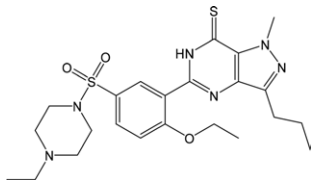
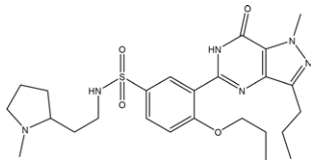
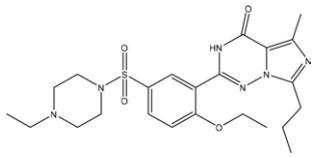
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Lodenafil carbonate	398507-55-6	$C_{47}H_{62}N_{12}O_{11}S_2$	Target panel	
Mirodenafil	862189-95-5	$C_{26}H_{37}N_5O_5S$	Target panel	
Propoxyphenyl homohydroxysildenafil	139755-87-6	$C_{24}H_{34}N_6O_5S$	Target panel	
Sildenafil	139755-83-2	$C_{22}H_{30}N_6O_4S$	Target panel	
Tadalafil	171596-29-5	$C_{22}H_{19}N_3O_4$	Target panel	
Thiohomosildenafil	479073-80-8	$C_{23}H_{32}N_6O_3S_2$	Target panel	
Udenafil	268203-93-6	$C_{25}H_{36}N_6O_4S$	Target panel	
Vardenafil	224785-90-4	$C_{23}H_{32}N_6O_4S$	Target panel	

Table 2. (*continued*)

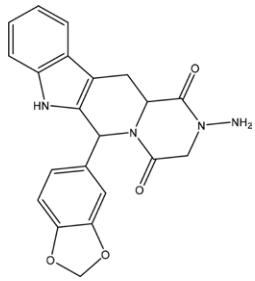
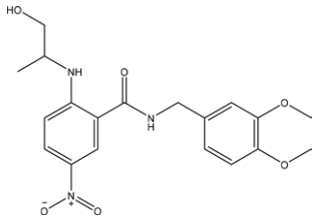
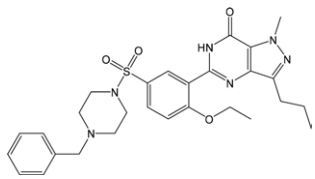
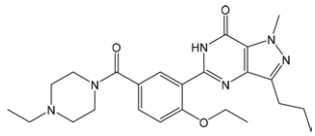
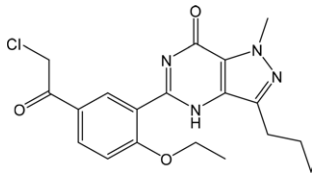
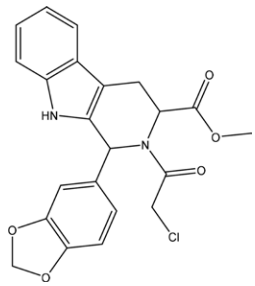
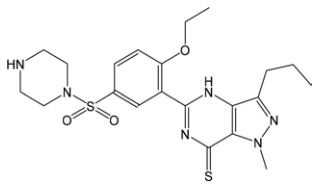
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Aminotadalafil	385769-84-6	$C_{21}H_{18}N_4O_4$	—[a]	
Benzamidenafil	1020251-53-9	$C_{19}H_{23}N_3O_6$	—	
Benzylsildenafil	1446089-89-2	$C_{28}H_{34}N_6O_4S$	—	
Carbodenafil	Not available	$C_{24}H_{32}N_6O_3$	—	
Chlorodenafil	1058653-74-9	$C_{19}H_{21}ClN_4O_3$	—	
Chloropretadalafil	171489-59-1	$C_{22}H_{19}ClN_2O_5$	—	
Desmethylthiosildenafil	479073-86-4	$C_{21}H_{28}N_6O_3S_2$	—	

Table 2. (*continued*)

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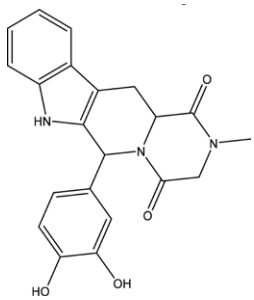
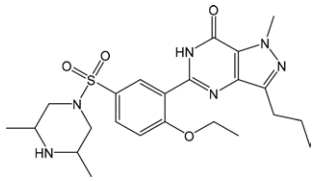
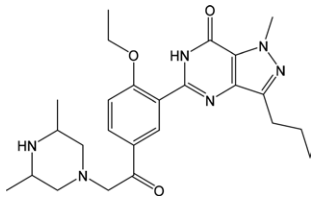
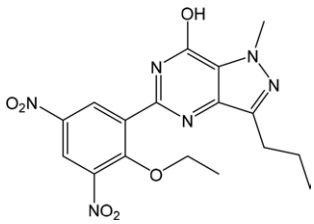
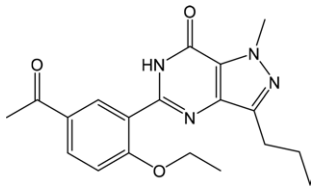
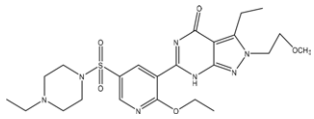
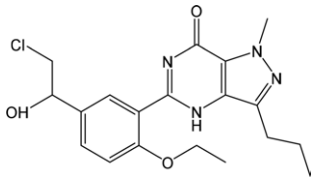
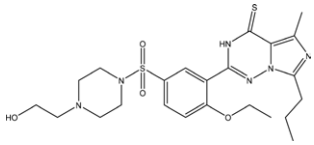
Desmethylenetadalafil	171489-03-5	$C_{21}H_{19}N_3O_4$	—	
Dimethylsildenafil	1416130-63-6	$C_{23}H_{32}N_6O_4S$	—	
Dimethylacetildenafil	Not available	$C_{25}H_{34}N_6O_3$	—	
Dinitrodenafil	Not available	$C_{17}H_{18}N_6O_6$	—	
Gendenafil	147676-66-2	$C_{19}H_{22}N_4O_3$	—	
Gisadenafil	334826-98-1	$C_{23}H_{33}N_7O_5S$	—	
Hydroxychlorodenafil	1391054-00-4	$C_{19}H_{23}ClN_4O_3$	—	
Hydroxythiovardenafil	912576-30-8	$C_{23}H_{32}N_6O_4S_2$	—	

Table 2. (*continued*)

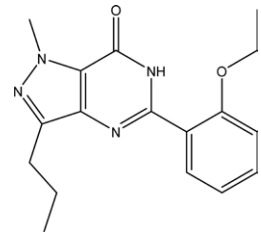
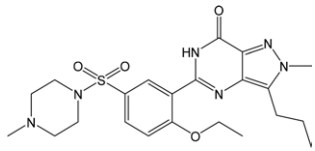
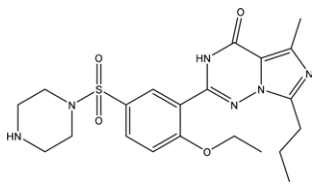
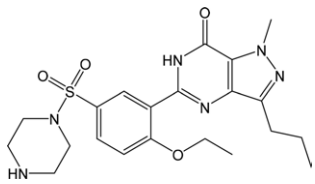
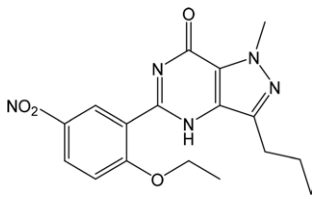
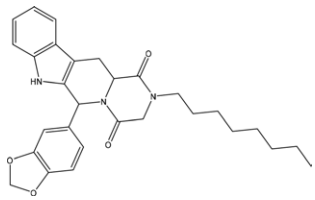
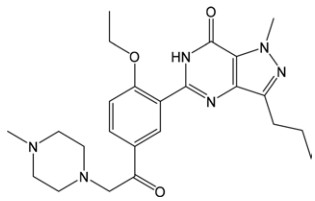
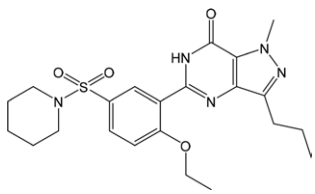
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Imidazosagatriazinone	139756-21-1	$C_{17}H_{20}N_4O_2$	—	
Isosildenafil	253178-46-0	$C_{22}H_{30}N_6O_4S$	—	
N-Desethyl vardenafil	448184-46-1	$C_{21}H_{28}N_6O_4S$	—	
N-Desmethyl sildenafil	139755-82-1	$C_{21}H_{28}N_6O_4S$	—	
Nitrodenafil	147676-99-1	$C_{17}H_{19}N_5O_4$	—	
N-Octyl nortadalafil	1173706-35-8	$C_{29}H_{33}N_3O_4$	—	
Noracetildenafil	949091-38-7	$C_{24}H_{32}N_6O_3$	—	
Norneosildenafil	371959-09-0	$C_{22}H_{29}N_5O_4S$	—	

Table 2. (*continued*)

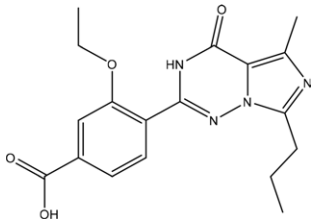
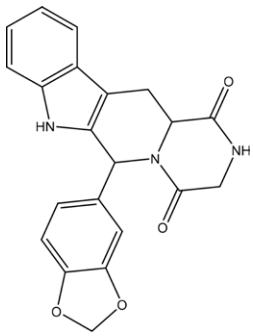
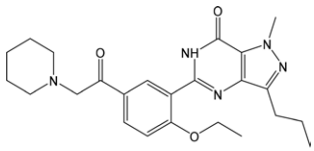
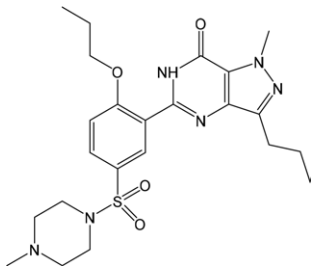
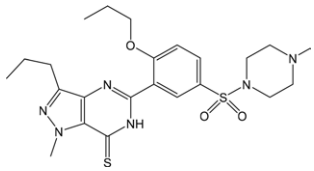
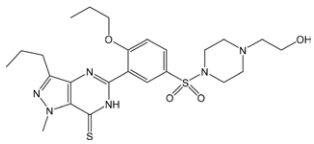
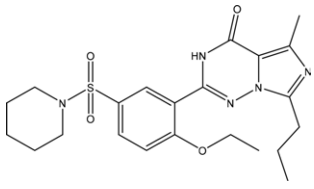
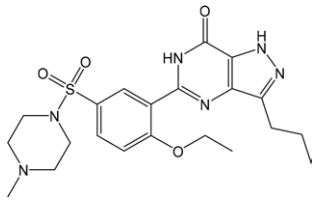
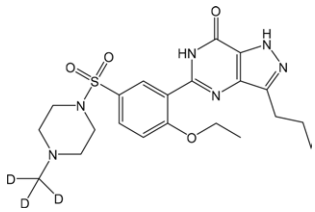
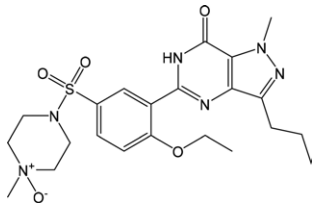
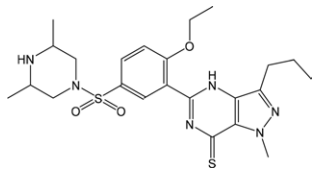
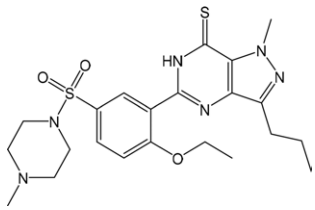
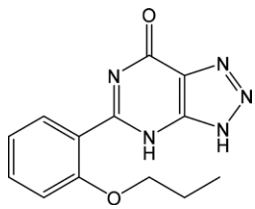
Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Norneovardenafil	358390-39-3	$C_{18}H_{20}N_4O_4$	—	
Nortadalafil	171596-36-4	$C_{21}H_{17}N_3O_4$	—	
Piperiacetildenafil	147676-50-4	$C_{24}H_{31}N_5O_3$	—	
Propoxyphenyl sildenafil	877777-10-1	$C_{23}H_{32}N_6O_4S$	—	
Propoxyphenyl thiosildenafil	479073-87-5	$C_{23}H_{32}N_6O_3S_2$	—	
Propoxyphenyl thiohydroxyhomosildenafil	479073-90-0	$C_{24}H_{34}N_6O_4S_2$	—	
Pseudovardenafil	224788-34-5	$C_{22}H_{29}N_5O_4S$	—	

Table 2. (*continued*)

Analyte	Chemical Abstracts Service No.	Formula	Note	Structure
Pyrazole *N*-demethyl sildenafil	139755-95-6	$C_{21}H_{28}N_6O_4S$	—	
Pyrazole *N*-demethyl sildenafil-d_3	Not available	$C_{21}H_{25}D_3N_6O_4S$	IS	
Sildenafil *N*-oxide	1094598-75-0	$C_{22}H_{30}N_6O_5S$	—	
Thioaildenafil	856190-47-1	$C_{23}H_{32}N_6O_3S_2$	—	
Thiosildenafil	479073-79-5	$C_{22}H_{30}N_6O_3S_2$	—	
Zaprinast	37762-06-4	$C_{13}H_{13}N_5O_2$	—	

[a] — = Additional evaluated analytes.

Table 3. Matrixes evaluated in the SLV study

Code	Form	Active ingredients	Other ingredients
M1	Powder	*Tribulus terrestris*	Not available
M2	Extract	*Epimedium*	Not available
M3	Softgel	Maca root powder, *Ashwagandha* powder, *Epimedium* extract, *Tribulus* extract, Yohimbe bark extract, ginger root extract, long pepper fruit extract, black pepper fruit extract	Soybean oil, gelatin, glycerin, purified water, beeswax, Soy lecithin, caramel color
M4	Liquid	Damiana leaf extract, ginseng root extract, saw palmetto, *Tribulus terrestris* fruit extract, *Avena sativa* extract, bee pollen extract, guarana seed extract, Yohimbe bark extract, royal jelly	Distilled water, glycerin
M5	Capsule	Maca powder, Horny goat weed extract, *Tribulus* extract, Yohimbe extract, cayenne extract, Asian ginseng extract, ginger extract, long pepper extract, black pepper extract	Gelatin, silica, vegetable stearate
M6	Tablet	*Pinus pinaster* bark extract, *Epimedium sagittatum* extract	Corn starch, maltodextrin, cellulose, vegetable stearate, silica, glycerin, purified water
M7	Liquid extract (tincture)	*Epimedium grandiflorum* dried leaves	Glycerine, alcohol 60%, distilled water

Canada), Cachesyn (Mississagua, Canada), TLC Pharmachem (Vaughan, Canada), and Sigma-Aldrich (St. Louis, MO).

D. Preparation of Reagent Solutions and Standards

(a) *50:50 (v/v) ACN:H_2O.*—Combine 500 mL HPLC grade ACN and 500 mL deionized H_2O. Sonicate for 2 min.

(b) *70:30 (v/v) H_2O:ACN.*—Combine 700 mL deionized H_2O and 300 mL HPLC grade ACN. Sonicate for 2 min.

(c) *LC mobile phase A.*—Weigh 0.63±0.01 g NH_4OFor in an appropriate reservoir and add 1000 mL H_2O and 1 mL FA. Mix thoroughly.

(d) *LC mobile phase B.*—Weigh 0.63±0.01 g NH_4OFor in an appropriate reservoir and add 500 mL MeOH. Sonicate for approximately 3 min. Add 500 mL ACN and 1 mL FA. Mix thoroughly.

(e) *Individual stock solutions.*—Prepare individual solutions of PDE5 inhibitors at concentrations ranging from 1500 to 4000 µg/mL. For aminotadalafil, benzyl sildenafil, chloropretadalafil, desmethylene tadalafil, lodenafil carbonate, tadalafil, and thioaildenafil use a mixture of MeOH and chloroform (2:1, v/v). For the remaining analytes, use MeOH. If needed, sonicate at approximately 30°C to allow for complete dissolution of the solid standard.

(f) *Mixed stock standard solution.*—Combine individual analyte stock solutions to prepare a composite solution at 20 µg/mL in MeOH.

(g) *Internal standard (IS) solution.*—Prepare a solution at 20 µg/mL in MeOH using a stock solution of pyrazole *N*-demethyl sildenafil-d_3.

(h) *QC solvent standard.*—Accurately transfer 125 µL of the mixed stock standard solution and 125 µL the IS solution into a 10 mL volumetric flask. Dilute to volume with 70:30 (v/v) H_2O:ACN solution.

E. Sample Preparation

(a) *Homogenization and storage of samples.*—Solid samples such as botanical powders, extracts, and tablets were blended to obtain homogeneity and stored at –4°C. Softgels, gelcaps, and capsules were homogenized using cryogenic grinding with liquid nitrogen and stored at –70°C. Liquid samples were briefly shaken and stored at –4°C.

(b) *Extraction procedure.*—(*1*) Weigh 1.00±0.02 g thoroughly homogenized sample in a 50 mL centrifuge tube.

(*2*) Add 20 mL 50:50 (v/v) ACN:H_2O solution, briefly hand shake/vortex, and then shake for 15 min using a horizontal shaker set at approximately 250 rpm.

(*3*) Centrifuge the tube at >3000 × *g* for 5 min.

(*4*) Transfer 1 mL supernatant to another 50 mL centrifuge tube.

Note: When transferring extract aliquots obtained for softgels, avoid the upper lipophilic layer that forms during the centrifugation step.

(*5*) Add 19 mL 70:30 (v/v) H_2O:ACN solution and briefly vortex mix.

(*6*) Filter approximately 3 mL diluted extract using a plastic syringe fitted with a 0.22 µm PFTE syringe filter into a 15 mL centrifuge tube.

(*7*) Transfer 1 mL filtrate to a 2 mL autosampler vial and add 12.5 µL IS solution.

(*8*) Cap the vial and briefly vortex mix.

(*9*) Perform LC-HRMS analysis.

F. LC-HRMS Analysis

(a) *LC operating conditions.*—(*1*) *Column.*—Thermo Scientific Accucore aQ, 2.6 µm, 100×2.1 mm.

(*2*) *Column temperature.*—30°C.

(*3*) *Mobile phase A.*—10 mM NH_4OFor and 0.1% FA in H_2O.

(*4*) *Mobile phase B.*—10 mM NH_4OFor and 0.1% FA in ACN–MeOH (50:50, v/v).

(*5*) *Flow rate.*—0.3 mL/min.

(*6*) *Elution gradient.*—*See* Table **2015.12A**.

(*7*) *Injection volume.*—3 µL.

(*8*) *Autosampler temperature.*—15°C.

(*9*) *Run time.*—25 min.

(b) *MS data acquisition and operating conditions.*—MS data acquisition is performed in full MS–data-dependent product ion scan (dd-MS^2) and all-ion fragmentation (AIF) modes using the parameter settings provided below. Data-dependent product ion scan experiment is initiated if a mass (*m/z*) specified in an inclusion list (*see* Table **2015.12A**) is detected in the correct retention time (RT) window within a mass error of 10 ppm and at an intensity above the set threshold level. The ion

Table 2015.12A. Gradient elution program

Time, min	A, %	B, %
0.00	98	2
0.50	98	2
2.00	60	40
20.00	5	95
23.00	5	95
23.01	98	2
24.00	98	2

fragmentation in AIF and dd-MS^2 modes is performed at three discrete normalized collision energy (NCE) values.

(*1*) *Ionization mode.*—positive ESI.
(*2*) *Sheath gas flow.*—35 arb.
(*3*) *Auxiliary gas flow.*—10 arb.
(*4*) *Sweep gas flow.*—1 arb.
(*5*) *Spray voltage.*—3.5 kV.
(*6*) *Capillary temperature.*—350°C.
(*7*) *S-lens RF level.*—50 V.
(*8*) *Auxiliary gas heater temperature.*—350°C.
(*9*) *Full MS resolution.*—70 000 full width at half-maximum (FWHM).
(*10*) *Full MS automatic gain control AGC target.*—1e6.
(*11*) *Full MS maximum injection time (IT).*—100 ms.
(*12*) *Full MS scan range.—m/z* 200–1100.
(*13*) *dd-MS2 resolution.*—17 500 FWHM.
(*14*) *dd-MS2 AGC target.*—1e5.
(*15*) *dd-MS2 isolation window.*—1.0 Da.
(*16*) *dd-MS2 stepped NCE.*—40, 70, 100%.
(*17*) *Intensity threshold.*—2.0e4.
(*18*) *Apex trigger.*—1 to 6 s
(*19*) *Dynamic exclusion.*—6 s
(*20*) *AIF resolution.*—70 000 FWHM.
(*21*) *AIF AGC target.*—1e6.
(*22*) *AIF maximum IT.*—100 ms.
(*23*) *AIF stepped NCE.*—40, 70, 100%.
(*24*) *AIF scan range.—m/z* 50–750.

(c) *Inclusion list.—See* Table **2015.12B**.

(d) *Positive and negative control.*—Analyze a reagent blank (a negative control) with each sample set. Inject the QC solvent standard (a positive control) at the beginning of the LC-HRMS sequence, after every 10 samples, and again at the end of the LC-HRMS sequence. The IS response in samples should be within 40–140% of its average response in the QC solvent standards.

G. Data Processing

(a) *Workflow and detection/identification criteria.*—Detection and identification of analytes was performed with TraceFinder software and the settings indicated below. Detection of targeted PDE5 inhibitors was based on the automatic comparison of peak RTs extracted the from full MS record and the accurate mass of respective pseudomolecular ions $[M+H]^+$ with information from the TraceFinder compound database (*see* Table **2015.12C**). An RT of 30 s and mass tolerances of 5 ppm were used. To identify an analyte, additional criteria must be fulfilled. These include mass accuracy ($\Delta\, m/z \leq 5$ ppm) and relative responses (10% tolerance) of pseudomolecular ion isotopes, as well as criteria for fragment ions detected in appropriate dd-MS^2 records. For positive identification, one or more fragment ions listed in the TraceFinder compound database must be detected above the intensity threshold with a mass error of ≤5 ppm. The detection/identification workflow for targeted compounds is provided in Figure **2015.12**. PDE5 inhibitors not included in the TraceFinder compound database can be detected and identified by extracting the respective pseudomolecular ions from the full MS records and evaluating fragment ions in AIF records. A search using common PDE5 inhibitor fragments can be used to highlight components with structures similar to known PDE5 inhibitors.

(b) *TraceFinder software settings.*—(*1*) *RT range.*—1–23 min.
(*2*) *Peak area threshold.*—100 000.
(*3*) *Signal-to-noise threshold.*—10.
(*4*) *Mass tolerance (parent ion).*—5 ppm.

Table 2015.12B. Inclusion list used in the dd-MS^2 experiment for the target compound panel

Mass, *m/z*	Chemical formula	Species	Charge state	Polarity	Start, min	End, min
483.27143	$C_{25}H_{34}N_6O_4$	+H	1	Positive	4.06	4.36
467.27652	$C_{25}H_{34}N_6O_3$	+H	1	Positive	4.35	4.65
489.22785	$C_{23}H_{32}N_6O_4S$	+H	1	Positive	4.72	5.02
505.22277	$C_{23}H_{32}N_6O_5S$	+H	1	Positive	4.86	5.16
484.18584	$C_{23}H_{26}ClN_7O_3$	+H	1	Positive	4.88	5.18
475.21220	$C_{22}H_{30}N_6O_4S$	+H	1	Positive	4.92	5.22
489.22785	$C_{23}H_{32}N_6O_4S$	+H	1	Positive	5.08	5.38
433.15065	$C_{23}H_{20}N_4O_5$	+H	1	Positive	5.18	5.48
517.25915	$C_{25}H_{36}N_6O_4S$	+H	1	Positive	5.73	6.03
519.23842	$C_{24}H_{34}N_6O_5S$	+H	1	Positive	5.80	6.10
390.14483	$C_{22}H_{19}N_3O_4$	+H	1	Positive	5.97	6.27
532.25882	$C_{26}H_{37}N_5O_5S$	+H	1	Positive	7.78	8.08
521.19992	$C_{23}H_{32}N_6O_4S_2$	+H	1	Positive	8.60	8.90
505.20501	$C_{23}H_{32}N_6O_3S_2$	+H	1	Positive	8.92	9.22
1035.41752	$C_{47}H_{62}N_{12}O_{11}S_2$	+H	1	Positive	12.78	13.08

Table 2015.12C. TraceFinder software compound database for the target compound panel

Compound name	Chemical formula	Extracted mass	Adduct	RT	Fragment ions, *m/z*
Acetaminotadalafil	$C_{23}H_{20}N_4O_5$	433.15065	M+H	5.33	204.08078; 262.08626; 135.04406; 205.08860; 233.08352; 232.07569; 169.07602; 191.07295; 263.09408; 250.08626
Acetildenafil	$C_{25}H_{34}N_6O_3$	467.27652	M+H	4.50	111.09167; 97.07602; 70.06513; 84.08078; 72.08078; 127.12297; 112.09950; 297.13460; 56.04948; 166.09749
Avanafil	$C_{23}H_{26}ClN_7O_3$	484.18584	M+H	5.03	155.02582; 375.12184; 105.03349; 77.03858; 95.04914; 53.03858; 357.11128; 233.10330; 67.05423; 221.10330
Homosildenafil	$C_{23}H_{32}N_6O_4S$	489.22785	M+H	5.23	72.08078; 58.06513; 99.09167; 113.10732; 70.06513; 283.11895; 84.08078; 71.07295; 114.11515; 311.15025
Hydroxyacetildenafil	$C_{25}H_{34}N_6O_4$	483.27143	M+H	4.21	97.07602; 70.06513; 127.08659; 143.11789; 100.07569; 297.13460; 88.07569; 166.09749; 112.09950; 128.09441
Hydroxyhomosildenafil	$C_{23}H_{32}N_6O_5S$	505.22277	M+H	5.01	99.09167; 70.06513; 58.06513; 84.06820; 97.07602; 283.11895; 88.07569; 129.10224; 112.0995; 311.15025
Hydroxythiohomo-sildenafil	$C_{23}H_{32}N_6O_4S_2$	521.19992	M+H	8.75	99.09167; 70.06513; 58.06513; 84.06820; 299.09611; 129.10224; 97.07602; 88.07569; 327.12741; 112.09950
Lodenafil carbonate	$C_{47}H_{62}N_{12}O_{11}S_2$	1035.41752	M+H	12.93	112.09950; 82.06513; 97.07602; 111.09167; 487.21220; 83.06037; 84.08078; 283.11895
Mirodenafil	$C_{26}H_{37}N_5O_5S$	532.25882	M+H	7.93	99.09167; 296.13935; 312.13427; 70.06513; 56.04948;84.06820; 210.06619; 129.10224; 88.07569; 121.03964
Propoxyphenyl homohydroxysildenafil	$C_{24}H_{34}N_6O_5S$	519.23842	M+H	5.95	99.09167; 70.06513; 283.11895; 84.06820; 97.07602; 299.11387; 129.10224; 88.07569; 112.09950; 255.12404
Sildenafil	$C_{22}H_{30}N_6O_4S$	475.2122	M+H	5.07	58.06513; 100.09950; 99.09167; 56.04948; 283.11895; 70.06513; 311.15025; 225.07709; 299.11387
Tadalafil	$C_{22}H_{19}N_3O_4$	390.14483	M+H	6.12	204.08078; 135.04406; 262.08626; 169.07602; 205.08860; 232.07569; 233.08352; 240.11314; 268.10805; 250.08626
Thiohomosildenafil	$C_{23}H_{32}N_6O_3S_2$	505.20501	M+H	9.07	72.08078; 99.09167; 113.10732; 56.04948; 299.09611; 70.06513; 84.08078; 327.12741; 71.07295; 355.15806
Udenafil	$C_{25}H_{36}N_6O_4S$	517.25915	M+H	5.88	84.08078; 112.11208; 283.11895; 58.06513; 325.16590; 299.11387; 81.06988; 255.124037; 79.05423; 82.06513
Vardenafil	$C_{23}H_{32}N_6O_4S$	489.22785	M+H	4.87	169.09715; 344.14791; 110.06004; 299.11387; 72.08078; 123.09167; 70.06513; 376.10740; 68.01309; 113.10732

(*5*) *RT tolerance.*—30 s.

(*6*) *Minimum No. of fragments.*—1.

(*7*) *Intensity threshold.*—1000.

(*8*) *Mass tolerance (fragment ion).*—5 ppm.

(*9*) *Isotope pattern fit threshold.*—95%.

(*10*) *Mass tolerance (isotope).*—5 ppm.

(*11*) *Intensity tolerance (isotope).*—10%.

(c) *TraceFinder compound database.*—The compound database (*see* Table **2015.12C**) comprises information on the exact mass of pseudomolecular ions, molecular formulas, and RTs and the exact masses for 8–10 fragment ions for each analyte. The *m/z* values of fragments in the compound database represent exact masses that were calculated using experimental data obtained by HRMS analysis of reference standards and elucidation of fragment ions in Mass Frontier (Thermo Fisher Scientific, San Jose, CA) spectral interpretation software or based on information available in mzCloud database (Thermo Fisher Scientific, San Jose, CA) and scientific literature.

Results and Discussion

Chromatographic Separation

PDE5 inhibitors have multiple basic nitrogen groups in their molecules, which makes them prone to pH-dependent chromatographic issues, such as tailing or poor peak shape caused by the presence of analytes in both neutral and ionized forms. The mobile phase composition was optimized to minimize/eliminate these problems by using 10 mM ammonium formate and 0.1% FA in both mobile phases A and B. Addition of the acid to the mobile phase was essential to obtaining a good peak shape for norneovardenafil, which has an acidic carboxyl group in its molecule.

The composition of the organic mobile phase component had a significant impact on the chromatographic resolution between several isobaric compounds. Because some of these analytes cannot be differentiated based on their MS fragmentation patterns, their sufficient chromatographic separation is critical for reliable identification. Best results were obtained when a

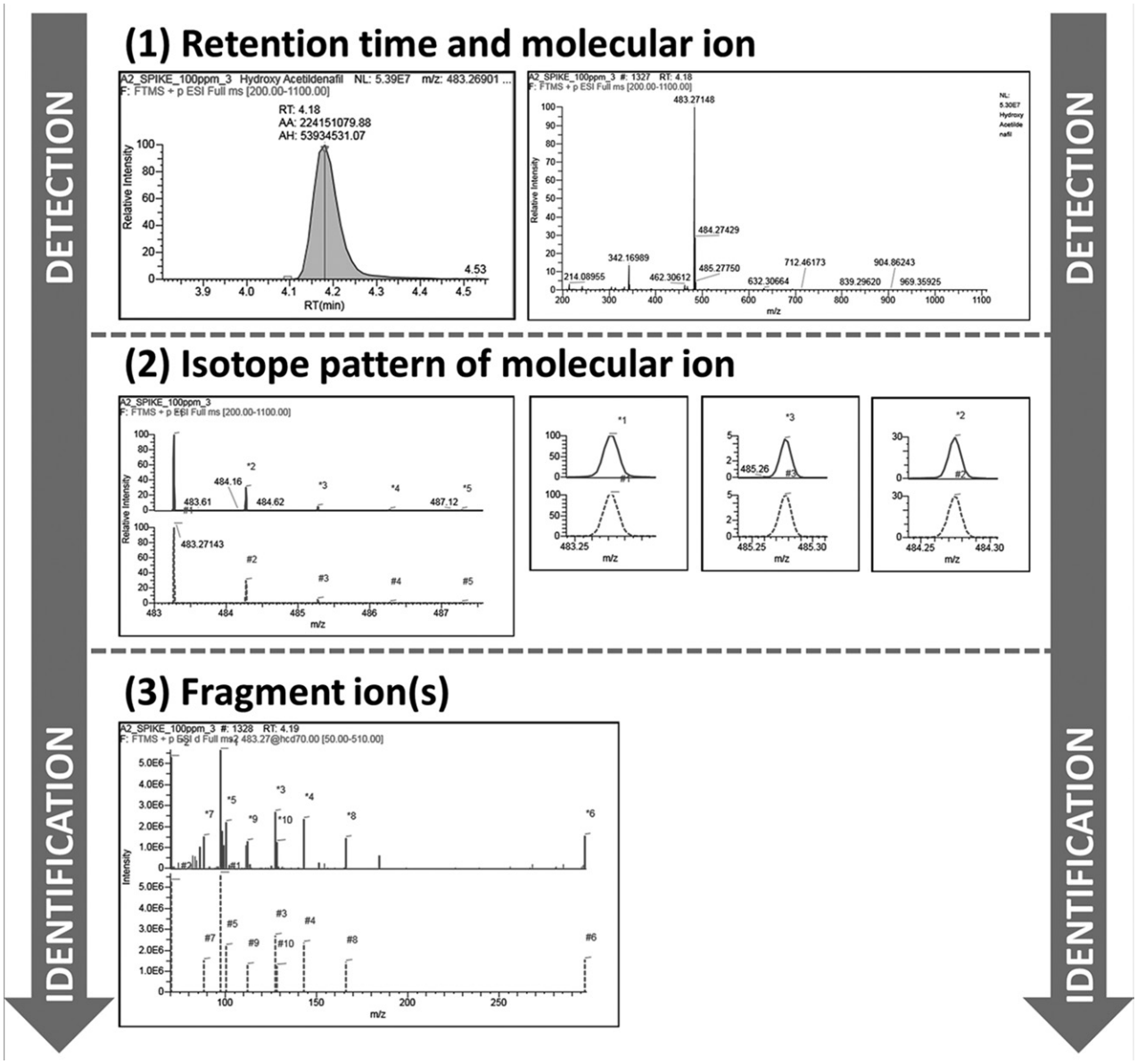


Figure 2015.12. Detection/identification workflow for targeted analytes.

mixture containing equal amounts of MeOH and ACN was used as the organic component of the mobile phase (*see* Figure 1). Under optimized conditions, analytes eluted between 3 and 15 min of the run with typical at-base peak widths ranging from 12 to 18 s. Of eight isobaric analyte groups, each containing two to four compounds, all analytes could be chromatographically resolved.

MS/MS Spectra

The availability of MS/MS data are crucial for reliable screening and identification of both known PDE5 inhibitors and their novel analogs. The MS/MS spectra of analytes were recorded in data-dependent product ion scan mode through the isolation and fragmentation of their respective pseudomolecular ions and in AIF mode. Rather than performing fragmentation at a single NCE setting, three discrete values of 40, 70, and 100% were used. This stepped NCE approach allowed obtaining fragments stable under different collision energies in a single MS experiment and resulted in information-rich MS/MS spectra.

Based on the review of the MS/MS spectra of all analytes, product ions frequently occurring in records of parent PDE5 inhibitors and their analogs were found. For example, fragment ion exact masses *m/z* 377.12780, 311.15025, 299.09611, 285.13460, 283.11895, and 99.09167 were frequently present in fragmentation spectra of sildenafil and its analogs, fragment *m/z* 204.08078 was characteristic of tadalafil and its analogs, and fragment ions *m/z* 123.09167 and 110.06004 were characteristic of vardenafil and its analogs. A combined

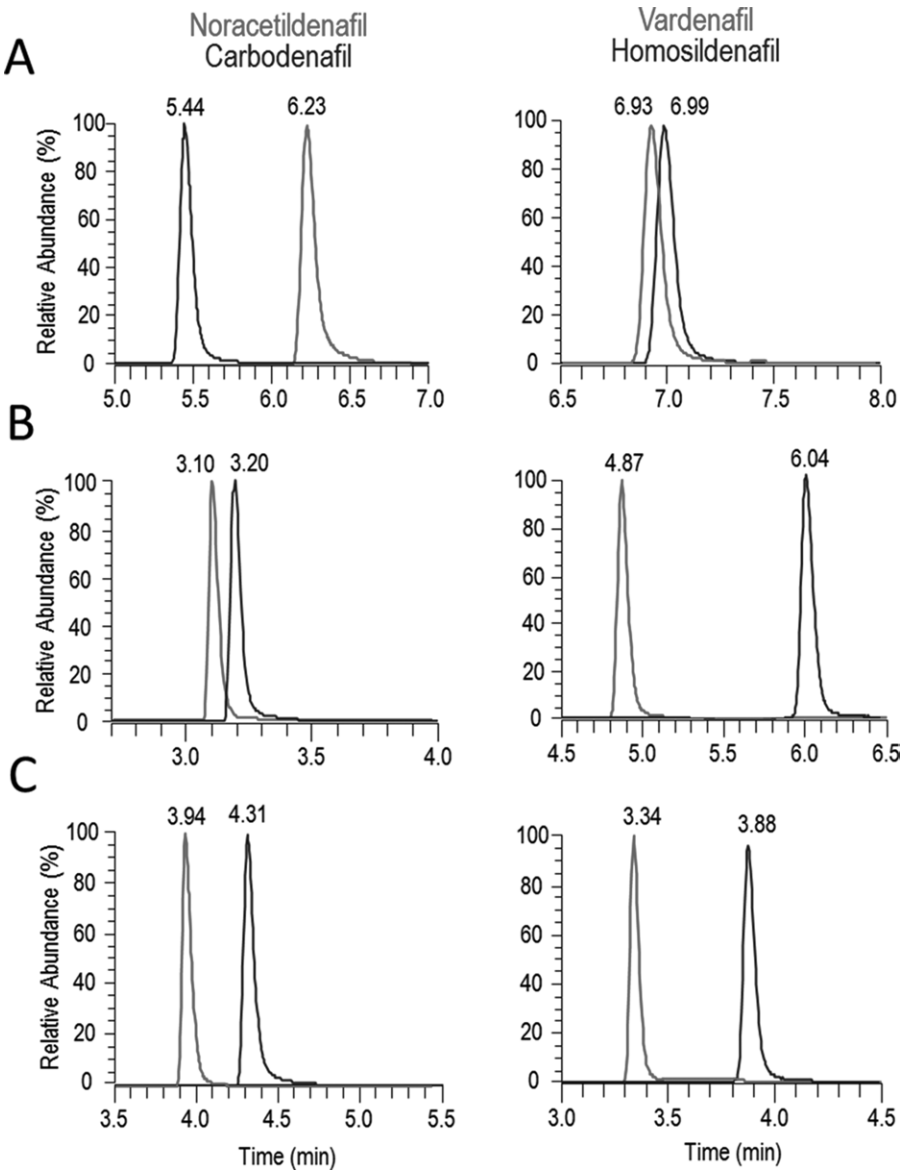


Figure 1. Impact of the mobile phase composition on peak shape and chromatographic resolution between isobaric analytes. (A) Mobile phase A/B: 0.1% FA in H_2O/0.1% FA in MeOH. (B) Mobile phase A/B: 5 mM ammonium formate in H_2O/5 mM ammonium formate in ACN. (C) Mobile phase A/B: 10 mM ammonium formate and 0.1% FA in H_2O/10 mM ammonium formate and 0.1% FA in ACN:MeOH (1:1, v/v).

search of these *m/z* values in AIF records can be used to detect nontargeted, novel PDE5 inhibitor adulterants based on their structural similarity to known PDE5 inhibitors.

Recovery and Repeatability

Results of recovery experiments conducted in triplicate at 50 mg/kg in a capsule sample in M5 are presented in Table 4. The test level of 50 mg/kg was selected for this evaluation to demonstrate the method performance at the target LOQ of AOAC SMPR 2014.011 for the determination of PDE5 inhibitors (6). The mean recoveries ranged from 69 to 90%. Only one target compound (thiohomosildenafil) was slightly below the recovery range of 70–120% provided in AOAC SMPR 2014.011. This method showed excellent repeatability with RSD_r values of 0.4–1.8%, well below the repeatability criteria of ≤20% in AOAC SMPR 2014.011.

POI

Detection and identification results are summarized in Table 5. In total, 1575 data points were evaluated to demonstrate POI and also probability of detection (POD) for detection/screening of PDE5 inhibitors. Correct detection/identification results compliant with identification requirements provided in the European Commission Decision 2002/657/EC (9) were obtained for all evaluated analytes at all concentration levels and in all matrixes. The method validation results fulfilled the

Table 4. Analyte recoveries and RSD_r obtained for the target compound panel in matrix M5 (capsule) at a spiking level of 50 mg/kg (n = 3)

Analyte	Mean recovery, %	RSD_r, %
Acetaminotadalafil	90	0.7
Acetildenafil	78	0.7
Avanafil	85	0.7
Homosildenafil	87	1.5
Hydroxyacetildenafil	79	1.8
Hydroxyhomosildenafil	88	1.1
Hydroxythiohomosildenafil	71	1.8
Lodenafil carbonate	83	1.6
Mirodenafil	85	0.8
Propoxyphenyl homohydroxysildenafil	85	0.7
Sildenafil	86	1.3
Tadalafil	90	0.4
Thiohomosildenafil	69	1.7
Udenafil	89	1.2
Vardenafil	83	2.2

POI requirements listed in AOAC SMPR 2014.010 and POD requirements (for the pooled data) listed in AOAC SMPR 2014.012.

Depending on the analyte and matrix type, 3–7 isotopic ions and 8–10 fragment ions in the raw data were typically matched with the information in the TraceFinder compound database. Excellent mass accuracy was obtained for pseudomolecular, isotopic, and fragment ions over a period of nearly 3 days of measurements with typical mass errors <1 ppm. Such stability of mass measurement was achieved with single-mass axis calibration of the instrument performed prior to starting the data acquisition. An example of chromatogram and product ion mass spectra obtained for sildenafil and tadalafil at 100 mg/kg (low test concentration) in matrix M1 (botanical powder) and their comparison with product ion spectra of reference standards are provided in Figure 2.

Figure 3 shows examples of chromatographic and mass spectral data obtained in matrix M1 at 100 mg/kg for two nontarget PDE5 inhibitors (*N*-octyl sildenafil and aminosildenafil) that were not included in the inclusion list or compound database. This demonstrates the method's ability to collect data for both targeted and nontargeted PDE5 inhibitors in a single chromatographic run. Detection and identification of nontarget PDE5 inhibitors and novel analogs are performed through the retrospective evaluation of MS and MS/MS experimental data. Common PDE5 inhibitor MS fragments can be used to detect compounds with structures similar to known PDE5 inhibitors and provide at least class identification in the cases of novel PDE5 inhibitor analogs, for which reference standards are not available.

Time-to-Result

Time-to-result for the analysis of one sample and detection/identification of PDE5 inhibitors included in the compound database were less than 1 h.

Conclusions

The SLV data demonstrate the acceptable performance of the presented method for screening and identification of both target and nontarget PDE5 inhibitors in dietary ingredients and supplements, meeting the requirements provided in AOAC SMPR **2014.010** and **2014.012**. The obtained recovery and repeatability results indicate that the method can be also used for quantification of PDE5 inhibitors. As discussed previously, only a reserved quantification evaluation was performed due to the limited availability and high cost of the reference standards required to spike samples at the high ppm levels.

Acknowledgments

The financial support through the Leeds City Region Enterprise Partnership Grant (BGP151COV) is gratefully acknowledged. This study was conducted in collaboration with Thermo Fisher Scientific. In particular, the authors thank the following people from Thermo Fisher Scientific in the United States and Europe: Charles Yang, Michael Hauer, Daniel Quinn, Frans Schoutsen, Michal Godula, Richard Fussell, and Dipankar Ghosh.

Table 5. Summary of the SLV results

Parameter	Test design	Target test concn, ppm	Correct detection results, %[a]	Correct identification results, %
POI at low concn	525 pooled data points, including evaluation of all target panel compounds in 7 matrix types with 5 replicates per matrix (35 samples and 15 analytes)	100	100	100
POI at high concn	525 pooled data points, including evaluation of all target panel compounds in 7 matrix types with 5 replicates per matrix (35 samples and 15 analytes)	1000	100	100
POI at 0 concn	525 pooled data points, including evaluation of all target panel compounds in 7 matrix types with 5 replicates per matrix (35 samples and 15 analytes)	0	100	100

[a] POD applies (AOAC SMPR 2014.012).

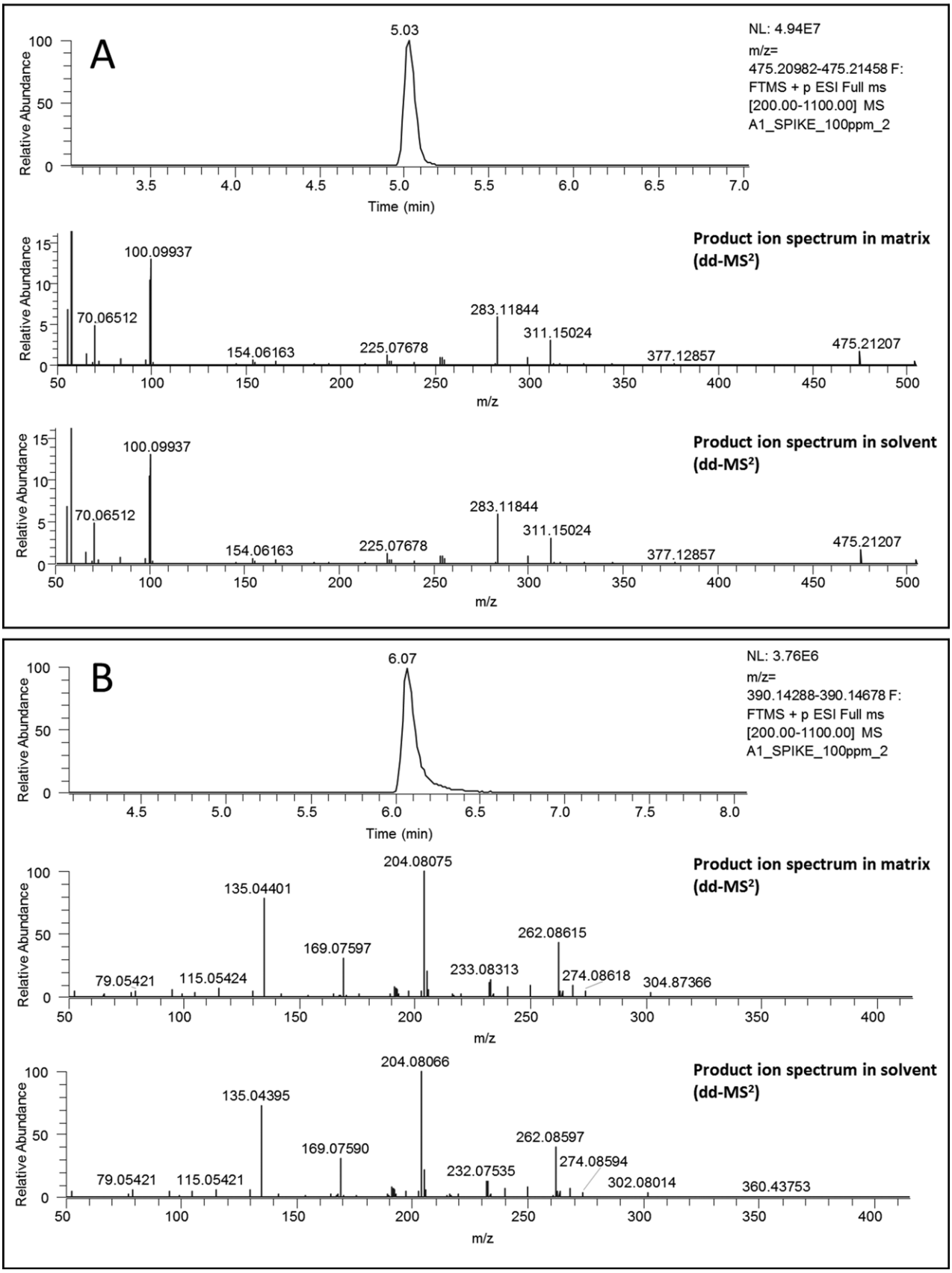


Figure 2. Chromatograms and mass spectra at 100 mg/kg (low test concentration) in matrix M1 (powder). (A) Sildenafil and (B) tadalafil.

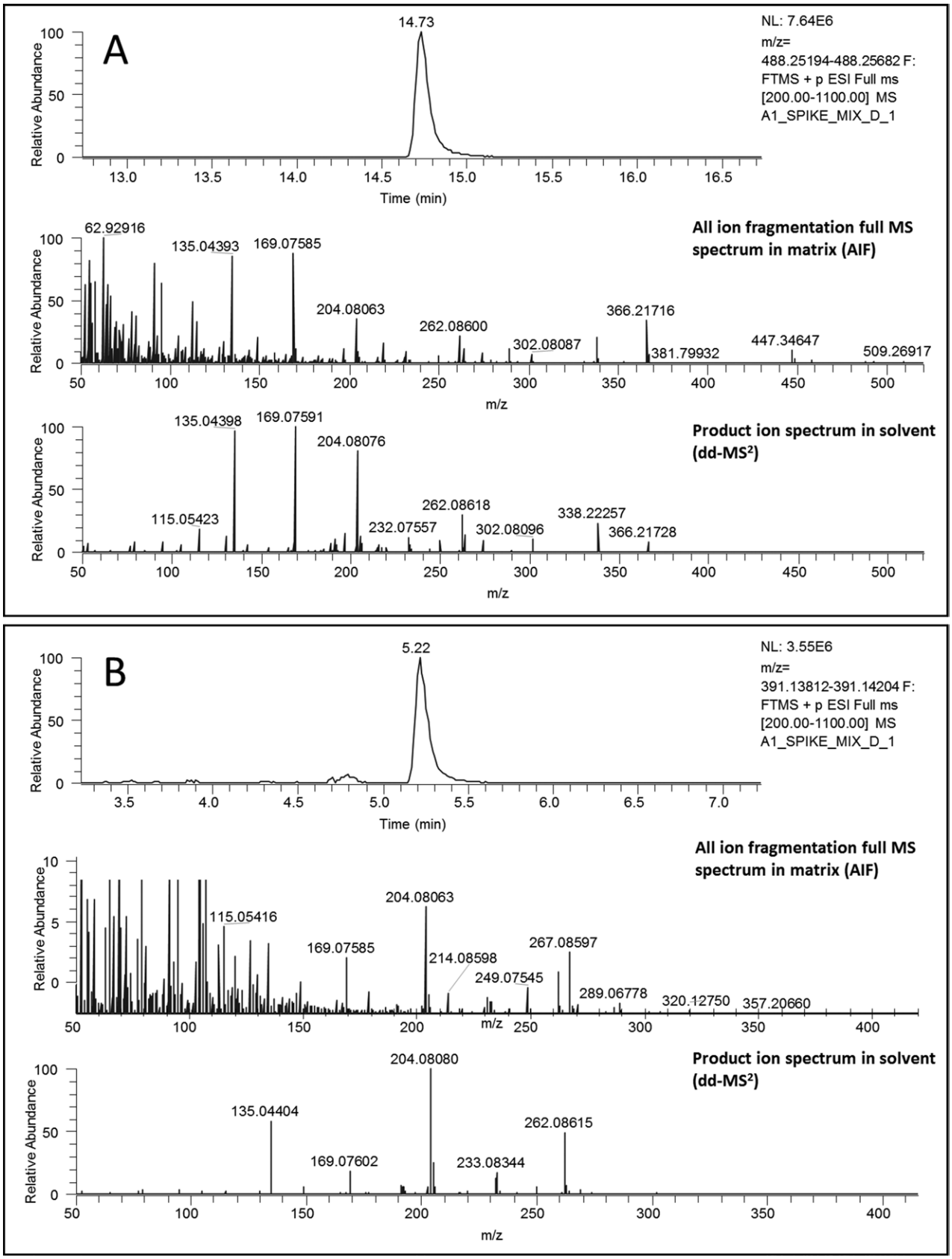


Figure 3. Examples of chromatograms and mass spectra obtained for selected nontarget analytes (not included in the inclusion list or compound database) at 100 mg/kg. (A) *N*-Octyl sildenafil and (B) aminotadalafil.

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17.2.11

AOAC Official Method 2015.13
Enumeration of Aerobic Bacteria in Food
3M™ Petrifilm™ Rapid Aerobic Count Plate
First Action 2015

[Applicable to the enumeration of aerobic bacteria from raw ground beef, raw ground pork, raw ground turkey, chicken carcass rinsate, fresh swai, fresh tuna, fresh tiger shrimp, raw easy-peel shrimp, cherry tomato wash, frozen blueberries, Mediterranean apricots, creamy salad dressing, fresh pasta, vanilla ice cream, instant nonfat dry milk (NFDM), and pasteurized skim milk.]

Caution: After use, the diluents and 3M Petrifilm RAC Plates may contain microorganisms that may be a potential biohazard. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal.

To reduce the risks associated with bacterial infection and workplace contamination: Perform 3M Petrifilm RAC Plate testing in a properly equipped laboratory under the control of a skilled microbiologist. The user must train personnel in current proper testing techniques; for example Good Laboratory Practices, ISO 17025, or ISO 7218.

See Tables **2015.13A** and **B** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

The 3M Petrifilm Rapid Aerobic Count (RAC) Plate is a sample-ready culture medium system which contains nutrients, a cold-water-soluble gelling agent, and an indicator system that facilitates aerobic bacterial enumeration. 3M Petrifilm RAC Plates are used for the enumeration of aerobic bacteria in as little as 24 h for most food matrices. 3M™ Food Safety is certified to ISO (International Organization for Standardization) 9001 for design and manufacturing.

B. Apparatus and Reagents

(**a**) *3M Petrifilm RAC Plate*.—25 plates/pouch, 2 pouches/box (3M Food Safety, St. Paul, MN, USA; Cat. No. 6478).

(**b**) *Sterile diluent.*—Butterfield's Phosphate Buffered Diluent.

(**c**) *Pipets.*—Capable of pipetting 1000 µL or a serological pipet.

(**d**) *Sterile pipet tips.*—Capable of 1000 µL.

(**e**) *Stomacher.*—Seward or equivalent.

(**f**) *Filter Stomacher bags.*—Seward or equivalent.

(**g**) *3M Petrifilm Flat Spreader.*—Cat. No. 6425.

(**h**) *Incubators.*—Capable of maintaining 32 ± 1°C and 35 ± 1°C and having a solid front to maintain a dark interior.

(**i**) *Refrigerator.*—Capable of maintaining 2–8°C, for storing the 3M Petrifilm RAC Plates.

(**j**) *Freezer.*—Capable of maintaining –10 to –20°C for storing 3M Petrifilm RAC pouches after incubation.

(**k**) *Standard Colony Counter or Illuminated Magnifier.*

C. General Instructions

(**a**) *Storage conditions.*—Store the 3M Petrifilm RAC Plates at 2–8°C. After opening the 3M Petrifilm RAC Plate pouches, seal the pouch and store at ambient temperature, less than 60% relative humidity. Post-incubation 3M Petrifilm RAC Plates can be stored at –10 to –20°C for up to 1 week.

(**b**) *Spreader.*—Place the 3M Petrifilm Flat Spreader on the center of the plate when preparing sample aliquot to prevent trapping air bubbles.

(**c**) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

D. Sample Preparation

(*1*) Aseptically prepare a 1:10 dilution of each test portion. *Dairy products.*—Pipet 11 mL or weigh 11 g of sample into 99 mL sterile Butterfield's Phosphate Buffered Diluent. *All other foods.*—Weigh a 50 g test portion into a sterile stomacher bag and dilute with 450 mL Butterfield's Phosphate Buffered Diluent; blend or homogenize per standard.

(*2*) Prepare 10-fold serial dilutions in Butterfield's Phosphate Buffered Diluent.

(*3*) Place two 3M Petrifilm RAC Plates on a flat, level surface for each dilution to be tested.

(*4*) Lift the film. With the pipet perpendicular dispense 1 mL of each dilution onto the center of the bottom film of each plate.

(*5*) Roll the film down onto the sample.

(*6*) Place the 3M Petrifilm Flat Spreader on the center of the plate. Press gently on the center of the spreader to distribute the

Table 2015.13A. Interlaboratory study results of 3M Petrifilm RAC Plate vs FDA BAM Chapter 3 method for raw easy-peel shrimp

	3M Petrifilm RAC Plate					FDA BAM Chapter 3								
Matrix	Lot	*N*[a]	Mean $\log_{10}$ CFU/g	s_r[b]	s_R[c]	Lot	*N*	Mean $\log_{10}$ CFU/g	s_r	s_R	Difference of means	Difference of means 95% LCL, UCL[d,e]	Reverse transformed difference of mean, CFU/g	Reverse transformed difference of means LCL, UCL
Raw easy-peel shrimp 32°C	Low	16	2.96	0.132	0.280	Low	16	3.02	0.218	0.356	0.06	–0.11, 0.24	139.47	0.77, 1.72
	Medium	16	4.29	0.202	0.215	Medium	16	4.23	0.095	0.298	–0.06	–0.18, 0.06	–2424.10	0.67, 1.15
	High	16	5.56	0.110	0.248	High	16	5.76	0.097	0.214	0.20	–0.01, 0.42	214352.79	0.97, 2.61
Raw easy-peel shrimp 35°C	Low	16	2.80	0.121	0.335	Low	16	3.02	0.218	0.356	0.22	–0.03, 0.48	422.68	0.92, 3.03
	Medium	16	4.22	0.172	0.273	Medium	16	4.23	0.095	0.298	0.01	–0.08, 0.11	539.37	0.83, 1.28
	High	16	5.67	0.141	0.174	High	16	5.76	0.097	0.214	0.09	–0.09, 0.26	105217.30	0.82, 1.83

[a] *N* = Number of laboratories that reported complete results.

[b] s_r = Repeatability.

[c] s_R = Reproducibility.

[d] LCL, UCL = 95% lower and upper confidence limits, respectively.

[e] A 95% confidence interval that contains the point 0 indicates no statistical significant difference between methods.

Table 2015.13B. Interlaboratory study results of 3M Petrifilm RAC Plate vs SMEDP Chapter 6 method for pasteurized skim milk and instant NFDM

	3M Petrifilm RAC Plate					SMEDP Chapter 6								
Matrix	Lot	N^a	Mean $\log_{10}$ CFU/g	s_r^b	s_R^c	Lot	N	Mean $\log_{10}$ CFU/g	s_r	s_R	Difference of means	Difference of means 95% LCL, UCL[d,e]	Reverse transformed difference of mean, CFU/g	Reverse transformed difference of means LCL, UCL
Pasteurized skim milk	Low	13	2.51	0.131	0.310	Low	13	2.47	0.123	0.301	–0.04	–0.08, 0.01	24.56	0.83, 1.03
	Medium	13	3.53	0.180	0.242	Medium	13	3.48	0.119	0.264	–0.05	–0.13, 0.03	346.20	0.75, 1.08
	High	13	4.63	0.136	0.232	High	13	4.58	0.116	0.196	–0.05	–0.11, 0.01	4936.41	0.78, 1.00
Instant NFDM	Low	15	2.42	0.096	0.126	Low	15	2.34	0.129	0.179	–0.08	–0.16, 0.01	42.05	0.69, 1.02
	Medium	15	3.04	0.059	0.148	Medium	15	2.98	0.104	0.195	–0.06	–0.14, 0.01	153.18	0.73, 1.02
	High	15	4.26	0.174	0.190	High	15	4.19	0.185	0.197	–0.07	–0.14, 0.01	2806.94	0.71, 1.00

[a] N = Number of laboratories that reported complete results.

[b] s_r = Repeatability.

[c] s_R = Reproducibility.

[d] LCL, UCL = 95% lower and upper confidence limits, respectively.

[e] A 95% confidence interval that contains the point 0 indicates no statistical significant difference between methods.

sample evenly. Spread the inoculum over the entire 3M Petrifilm RAC Plate growth area before the gel is formed. Do not slide the spreader across the film.

(*7*) Remove the spreader and leave the plate undisturbed for at least 1 min to permit the gel to form.

(*8*) Incubate the 3M Petrifilm RAC Plates at either 32 ± 1°C (seafood and dairy products) or 35 ± 1°C (all other foods) in a horizontal position with the clear side up in stacks of no more than 20 (dairy products) or 40 for all other foods. Enumerate plates after 24 ± 2 h of incubation (or 48 ± 3 h in the case of dairy powders including whey powder). 3M Petrifilm RAC Plates can be counted using a standard colony counter with the use of a back light or an illuminated magnifier to assist with the estimated enumeration.

(*9*) Enumerate all colonies, regardless of size, color, or intensity.

(*10*) The circular growth area is approximately 30 cm^2. Plates containing greater than 300 colonies can be either estimated or recorded as Too Numerous To Count (TNTC). Estimation can only be done by counting the number of colonies in one or more representative squares and determining the average number per square. The average number can be multiplied by 30 to determine the estimated count per plate. If a more accurate count is required, the sample may need to be retested at higher dilutions.

(*11*) Average the counts between the replicate plates. Report final results as colony forming units/gram (CFU/g or CFU/mL).

Note: If there are two dilutions within the countable range, use the following calculation to determine the final count:

$$N = \Sigma C/(1.1*d)$$

where N = number of colonies per mL/g of product; ΣC = sum of all colonies on both plates; and d = dilution from which first counts were obtained.

(*12*) Food samples may occasionally show interference on the 3M Petrifilm RAC Plates, for example:

(*a*) Uniform blue background color (often seen from the organisms used in cultured products) these should not be counted as TNTC.

(*b*) Intense pinpoint blue specs (often seen with spices or granulated products).

(*13*) When necessary, colonies may be isolated for further identification. Lift the top film and pick the colony from the gel.

Reference: *J. AOAC Int*. (future issue)

FOOD BIOLOGICAL CONTAMINANTS

Evaluation of the 3M™ Petrifilm™ Rapid Aerobic Count Plate for the Enumeration of Aerobic Bacteria: Collaborative Study, First Action 2015.13

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The 3M™ Petrifilm™ Rapid Aerobic Count (RAC) Plate is a sample-ready culture medium system containing dual-sensor indicator technology for the rapid quantification of aerobic bacteria in food products. The 3M Petrifilm RAC Plate was compared to the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (FDA BAM) Chapter 3 (Aerobic Plate Count) for the enumeration of aerobic bacteria in raw easy-peel shrimp and the *Standard Methods for the Examination of Dairy Products* (SMEDP) Chapter 6 (Standard Plate Count Method) for the enumeration of aerobic bacteria in pasteurized skim milk and instant nonfat dry milk (instant NFDM). The 3M Petrifilm RAC Plate was evaluated using a paired study design in a multilaboratory collaborative study following current AOAC validation guidelines. Three target contamination levels (low, 10–100 CFU/g; medium, 100–1000 CFU/g; and high 1000–10 000 CFU/g) were evaluated for naturally occurring aerobic microflora for each matrix. For raw easy-peel shrimp, duplicate 3M Petrifilm RAC Plates were enumerated after 24 ± 2 h incubation at both 32 and 35°C. Pasteurized skim milk 3M Petrifilm RAC Plates were enumerated after 24 ± 2 h incubation at 32°C, and instant NFDM 3M Petrifilm RAC Plates were enumerated after 48 ± 3 h incubation at 32°C. No statistical difference was observed between 3M Petrifilm RAC Plate and FDA BAM or SMEDP reference methods for each contamination level.

Submitted for publication January 6, 2016.
Corresponding author's email: pbird@qlaboratories.com
This method was approved by the Expert Review Panel for Microbiology Methods for Food and Environmental Surfaces as First Action.

The Expert Review Panel for Microbiology Methods for Food and Environmental Surfaces invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

DOI: 10.5740/jaoacint.15-0260

Aerobic plate counts are often used in the food industry to gauge the sanitary quality of food commodities throughout the production process, starting from raw materials used as ingredients to the shelf-life stability of finished products (1). Although generally not used as a safety indicator in food products, aerobic plate count can be useful in providing information on the deficiencies in sanitation systems or flaws in process control systems (2). This test can also be useful in providing information on the sanitary conditions of storage and processing facilities (1). Cultural enumeration of aerobic microflora can take 48–72 h, depending on the matrix, and can produce wide-ranging results as a result of difficulties that arise in the discernment of bacterial counts from matrix particulate. The 3M™ Petrifilm™ Rapid Aerobic Count (RAC) Plate uses a cold-water-soluble gelling agent and dual-sensing indicator technology to facilitate the enumeration of aerobic bacteria after 24 h of incubation for most foods (48 h for dairy powders, including whey powders).

The 3M Petrifilm RAC Plate allows for the simple and rapid enumeration of aerobic bacteria in the food and beverage industries. Samples are diluted in Butterfield's phosphate diluent (BPD) and a sample aliquot is plated onto the 3M Petrifilm RAC Plate. The sample aliquot is dispersed throughout the growth area and the plates can be incubated at either 32 or 35°C, depending on the matrix. Enumeration of colonies can occur in as little as 24 h of incubation for most matrixes. Enumeration is made easier due to the indicators in the gelling agent that use color to distinguish bacterial colonies from food particles.

Prior to the collaborative study, the 3M Petrifilm RAC Plate was validated according to AOAC validation guidelines (3) in a harmonized AOAC *Performance Tested Method*^{SM} (PTM) study. The objective of the PTM study was to demonstrate that the 3M Petrifilm RAC Plate accurately enumerated aerobic bacteria in select foods as claimed by the manufacturer and that no difference in repeatability was observed between the 3M Petrifilm RAC Plate method and the reference methods. For the 3M Petrifilm RAC Plate PTM evaluation, 16 matrixes were evaluated: raw ground beef, raw ground pork, raw ground turkey, chicken carcass rinsate, fresh swai, fresh tuna, fresh tiger shrimp, raw easy-peel shrimp, cherry tomato wash, frozen blueberries, dried Mediterranean apricots, creamy salad dressing, fresh pasta, vanilla ice cream, instant nonfat dry milk (NFDM), and pasteurized skim milk.

Additional PTM parameters (ruggedness, stability, and lot-to-lot variability) tested in the PTM studies satisfied the requirements for PTM approval. The method was awarded PTM certification No. 121403 on December 29, 2014.

The purpose of this collaborative study was to compare the 3M Petrifilm RAC Plate to the U.S. Food and Drug Administration (FDA) *Bacterial Analytical Manual* (BAM) Chapter 3 (Aerobic Plate Count; 4) and the Standard Methods for the Examination of Dairy Products (SMEDP) Chapter 6 (Standard Plate Count; 5), using BPD as the diluent for raw easy-peel shrimp, pasteurized skim milk, and instant NFDM.

Collaborative Study

Study Design

In this collaborative study, three matrixes—raw easy-peel shrimp, pasteurized skim milk, and instant NFDM—were evaluated. The matrixes were obtained from local retailers and screened for the presence of naturally occurring aerobic organisms by BAM or the SMEDP reference methods. Three separate levels of contamination were targeted for the evaluation of each matrix using naturally occurring aerobic microflora. The target levels for the naturally contaminated matrixes were low (10–100 CFU/g), medium (100–1000 CFU/g), and high (1000–10 000 CFU/g). To obtain the required contamination levels, bulk lots of the target matrixes were temperature-abused by heat-stressing to elevate the naturally occurring aerobic bacteria present in the matrixes, or diluted using lots containing low numbers of aerobic bacteria. Two replicate samples from each of the three contamination levels were analyzed by both candidate and reference methods in a paired study design. One set of paired samples (six total) per matrix was sent to each laboratory for analysis by the 3M Petrifilm RAC Plate and BAM or SMEDP reference methods.

A detailed collaborative study packet outlining all necessary information related to the study including media preparation, test portion preparation, and documentation of results was sent to each collaborating laboratory prior to the initiation of the study. A conference call was conducted prior to the initiation of the study to discuss the collaborative study packet and answer any questions from the participating laboratories.

Preparation of the Test Portions

For raw easy-peel shrimp and pasteurized skim milk test portions, a single bulk lot of the matrix was evaluated for total aerobic plate count following BAM or SMEDP, respectively, to determine baseline aerobic bacterial counts. For both raw easy-peel shrimp and pasteurized skim milk, two 1000 g portions were removed from the bulk lot and temperature-abused to increase the aerobic bacterial counts. For raw easy-peel shrimp, one set of 1000 g was placed in a 35 ± 1°C incubator for 1 h, and the second set of 1000 g was placed at room temperature (20–25°C) for 4 h. For pasteurized skim milk, one set of 1000 g was placed at room temperature (24 ± 2°C) for 4 h, and the second set of 1000 g was placed at room temperature (24 ± 2°C) for 12 h. Following temperature abuse, five replicate test portions from each lot were evaluated for total aerobic count using the specified reference method. Results from both test matrixes indicated that the temperature abuse had increased aerobic bacterial counts to produce two additional levels of contamination. The raw easy-peel shrimp samples were subsampled into 60 g test portions and the pasteurized skim milk samples were subsampled into 15 mL test portions to be sent to collaborators for use in the evaluation.

For instant NFDM, several lots of product were evaluated for the presence of aerobic bacteria. Initial testing identified two lots (one that produced aerobic plate counts in the high contamination level and one that produced counts in the low contamination level) to be used in the evaluation. The medium contamination level was prepared by mixing a portion of the high contamination lot with the low contamination lot. The instant NFDM samples were subsampled into 15 g test portions to be used in the evaluation.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded three-digit number affixed to the sample container. Test portions were shipped in leak-proof insulated containers via overnight delivery according to the Category B Dangerous Goods Regulations (DGR) as set forth by the International Air Transport Association (56th edition). Raw easy-peel shrimp and pasteurized skim milk samples were packed with cold packs to ensure refrigeration temperature (2–8°C) during shipment. Upon receipt, these test portions were held at refrigeration (2–8°C) until analyses were initiated the following day. Instant NFDM samples were packed and shipped at ambient temperature (24 ± 2°C). Upon receipt, samples were held at room temperature (24 ± 2°C) until analysis was initiated. In addition to each of the test portions, collaborators also received a test portion for each matrix labeled as "temperature control" for all three matrixes. Participants were instructed to record the temperature of this portion upon receipt of the shipment, document results on the Sample Receipt Confirmation form provided, and fax to the study director.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method specified for each matrix. For all three matrixes, each collaborator received six test portions (two high, two medium, and two low). For the analysis of the raw easy-peel shrimp by the 3M Petrifilm RAC Plate, a 50 g test portion was diluted with 450 mL BPD and homogenized by blending for 2 min. For pasteurized skim milk, 11 g test portion was diluted with 99 mL BPD and homogenized by shaking 25 times in a 30 cm arc within 7 s. For instant NFDM, 11 g sample was added to 99 mL tempered BPD (40–45°C) ensuring that the entire sample was visibly dissolved throughout the diluent prior to homogenizing by shaking 25 times in a 30 cm arc within 7 s. Ten-fold serial dilutions of each sample were prepared for each matrix and a 1.0 mL aliquot of each dilution was plated onto 3M Petrifilm RAC Plates. For raw easy-peel shrimp, four replicate 3M Petrifilm RAC Plates were prepared for each dilution, with two plates being incubated at 32 ± 1°C for 24 ± 2 h and two plates being incubated at 35 ± 1°C for 24 ± 2 h. For the evaluation of the pasteurized skim milk, two replicate 3M Petrifilm RAC Plates for each dilution were prepared and incubated at 32 ± 1°C for 24 ± 2 h. For the evaluation of the instant NFDM, two replicate 3M Petrifilm RAC Plates for each dilution were prepared and incubated at 32 ± 1°C for 48 ± 3 h. Seafood test portions were evaluated at two temperatures (32 and 35°C) to allow end users the option to choose either temperature for incubation. After incubation, 3M Petrifilm RAC Plates were removed from the incubator and typical colonies (all colonies regardless of size, color, or intensity) were enumerated using a

standard colony counter. Plates containing >300 colonies were either estimated or recorded as too numerous to count (TNTC). Estimations were conducted by counting the number of colonies in two or more representative squares and determining the average number per square. The average was multiplied by 30 to determine the estimated count per plate.

All matrixes analyzed by the 3M Petrifilm RAC Plate were also analyzed using BAM (shrimp) or SMEDP (milk and NFDM) reference methods in a paired study design. Serial dilutions for each sample were plated in duplicate onto plate count agar (BAM) or standard methods agar (SMEDP). For raw easy-peel shrimp and pasteurized skim milk, agar plates were incubated for 48 ± 4 h at 35 ± 1°C or 32 ± 1°C, respectively. Instant NFDM agar plates were incubated for 72 ± 3 h at 32 ± 1°C. Typical colonies in the countable range (25–250) were enumerated using a standard colony counter.

Statistical Analysis

Each collaborating laboratory recorded the CFU/g results for the reference methods and the 3M Petrifilm RAC Plate on the electronic spreadsheet provided in the collaborator study outline. The data sheets were submitted to the study director at the end of each week of testing for analysis. The data from each duplicate set of plates were averaged. A logarithmic transformation of the averaged counts was conducted for data analysis. Outliers were identified using Cochran and Grubbs' tests. The differences of means, including 95% upper and lower confidence limits, were determined for each contamination level for each matrix (6). If the confidence interval (CI) for the difference of means passed through the point 0, there was no statistical difference between the two methods (7). The reversed transformed difference of means, with a 95% CI, and the repeatability (s_r) and reproducibility (s_R) of the 3M Petrifilm RAC Plate and reference methods were also determined (7).

AOAC Official Method 2015.13
Enumeration of Aerobic Bacteria in Food
3M Petrifilm Rapid Aerobic Count Plate
First Action 2015

(Applicable to the enumeration of aerobic bacteria from raw ground beef, raw ground pork, raw ground turkey, chicken carcass rinsate, fresh swai, fresh tuna, fresh tiger shrimp, raw easy-peel shrimp, cherry tomato wash, frozen blueberries, Mediterranean apricots, creamy salad dressing, fresh pasta, vanilla ice cream, instant NFDM, and pasteurized skim milk)

See Tables **2015.13A** and **2015.13B** for a summary of results of the collaborative study.

See Tables **2015.13C–G** for detailed results of the collaborative study.

A. Principle

The 3M Petrifilm RAC Plate is a sample-ready culture medium system that contains nutrients, a cold-water-soluble gelling agent, and an indicator system that facilitates aerobic bacterial enumeration. 3M Petrifilm RAC Plates are used for the enumeration of aerobic bacteria in as little as 24 h for most food matrixes. 3M Food Safety is certified to ISO 9001 for design and manufacturing.

B. Apparatus and Reagents

(a) *3M Petrifilm RAC Plate*.—25 plates per pouch, two pouches per box. Available from 3M Food Safety (St. Paul, MN 55144-1000; Cat. No. 6478).

(b) *Sterile diluent*.—Butterfield's phosphate-buffered diluent.

(c) *Pipets*.—Capable of pipetting 1000 µL or a serological pipet.

(d) *Sterile pipet tips*.—Capable of 1000 µL.

(e) *Stomacher*.—Seward or equivalent.

(f) *Filter stomacher bags*.—Seward or equivalent.

(g) *3M Petrifilm Flat Spreader (Cat. No. 6425).*

(h) *Incubators*.—Capable of maintaining 32 ± 1°C and 35 ± 1°C and having a solid front to maintain a dark interior.

(i) *Refrigerator or Freezer*.—Capable of maintaining temperature between –20 to 8°C for storing unopened 3M Petrifilm RAC Plates.

(j) *Freezer*.—Capable of maintaining temperature at less than −15°C for storing 3M Petrifilm RAC pouches after incubation.

(k) *Standard colony counter or illuminated magnifier.*

Table 2015.13A. Interlaboratory study results of 3M Petrifilm RAC Plate vs FDA BAM Chapter 3 method for raw easy-peel shrimp

Matrix raw easy-peel shrimp	3M Petrifilm RAC Plate					FDA BAM Chapter 3						Difference of means[d]		
	Lot	N[a]	Mean $\log_{10}$ CFU/g	s_r[b]	s_R[c]	Lot	N	Mean $\log_{10}$ CFU/g	s_r	s_R	Difference of means	95% LCL, UCL	Reverse-transformed difference of the mean, CFU/g	Reverse-transformed difference of means LCL, UCL
32°C	Low	16	2.96	0.132	0.280	Low	16	3.02	0.218	0.356	0.06	–0.11, 0.24	139.47	0.77, 1.72
	Medium	16	4.29	0.202	0.215	Medium	16	4.23	0.095	0.298	–0.06	–0.18, 0.06	–2424.10	0.67, 1.15
	High	16	5.56	0.110	0.248	High	16	5.76	0.097	0.214	0.20	–0.01, 0.42	214352.79	0.97, 2.61
35°C	Low	16	2.80	0.121	0.335	Low	16	3.02	0.218	0.356	0.22	–0.03, 0.48	422.68	0.92, 3.03
	Medium	16	4.22	0.172	0.273	Medium	16	4.23	0.095	0.298	0.01	–0.08, 0.11	539.37	0.83, 1.28
	High	16	5.67	0.141	0.174	High	16	5.76	0.097	0.214	0.09	–0.09, 0.26	105217.30	0.82, 1.83

[a] Number of laboratories that reported complete results.

[b] s_r = Repeatability.

[c] s_R = Reproducibility.

[d] 95% lower and upper confidence limits. A 95% CI that contains the point 0, indicates no statistical significant difference between methods.

Table 2015.13B. Interlaboratory study results of 3M Petrifilm RAC Plate vs SMEDP Chapter 6 Method for pasteurized skim milk and instant NFDM

	3M Petrifilm RAC Plate					SMEDP Chapter 6						Difference of means[d]	Reverse-transformed difference of the means CFU/g	Reverse-transformed difference of means LCL, UCL
Matrix	Lot	N[a]		s_r[b]	s_R[c]	Lot	N	Mean Log_{10}, CFU/g	s_r	s_R	Difference of means	95% LCL, UCL		
Pasteurized skim milk	Low	13	2.51	0.131	0.310	Low	13	2.47	0.123	0.301	–0.04	–0.08, 0.01	24.56	0.83, 1.03
	Medium	13	3.53	0.180	0.242	Medium	13	3.48	0.119	0.264	–0.05	–0.13, 0.03	346.20	0.75, 1.08
	High	13	4.63	0.136	0.232	High	13	4.58	0.116	0.196	–0.05	–0.11, 0.01	4936.41	0.78, 1.00
Instant NFDM	Low	15	2.42	0.096	0.126	Low	15	2.34	0.129	0.179	–0.08	–0.16, 0.01	42.05	0.69, 1.02
	Medium	15	3.04	0.059	0.148	Medium	15	2.98	0.104	0.195	–0.06	–0.14, 0.01	153.18	0.73, 1.02
	High	15	4.26	0.174	0.190	High	15	4.19	0.185	0.197	–0.07	–0.14, 0.01	2806.94	0.71, 1.00

[a] Number of laboratories that reported complete results.

[b] s_r = Repeatability.

[c] s_R = Reproducibility.

[d] 95% lower and upper confidence limits. A 95% CI that contains the point 0, indicates no statistical significant difference between methods.

C. General Instructions

(a) *Storage conditions*.—Store the 3M Petrifilm RAC Plates at −20 to 8°C. After opening the 3M Petrifilm RAC Plate pouches, seal the pouch, and store at ambient temperature, <60% relative humidity. Post-incubation 3M Petrifilm RAC Plates can be stored at less than –15°C for up to 1 week.

(b) *Spreader*.—Place the 3M Petrifilm Flat Spreader on the center of the plate when preparing sample aliquot to prevent trapping air bubbles.

(c) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

Table 2015.13C. Participation of each collaborating laboratory[a]

Laboratory	Raw easy-peel shrimp	Skim milk	Instant NFDM
1	Y	Y	Y
2	Y	Y	Y
3	Y	Y	Y
4	Y	Y	Y
5	Y	Y	Y
6	Y	Y[b]	Y
7	Y	Y[b]	Y
8	Y	N	N
9	Y	Y	Y
10	Y	Y	Y
11	Y	Y	Y
12	Y	Y	Y
13	Y	Y	Y
14	Y	Y	Y
15	Y	Y	Y
16	Y	Y	Y

[a] Y = Collaborator analyzed the food type; N = collaborator did not analyze the food type.

[b] Results were not used in statistical analysis due to deviation of testing protocol or laboratory error.

Safety Precautions

After use, the diluents and 3M Petrifilm RAC Plates may contain microorganisms that may be a potential biohazard. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet (SDS) for additional information and local regulations for disposal. To reduce the risks associated with bacterial infection and workplace contamination, perform 3M Petrifilm RAC Plate testing in a properly equipped laboratory under the control of a skilled microbiologist. The user must train personnel in current proper testing techniques; for example Good Laboratory Practices, ISO 17025, or ISO 7218.

D. Sample Preparation

(a) Aseptically prepare a 1:10 dilution of each test portion.

(*1*) *Dairy products*.—Pipet 11 mL or weigh 11 g sample into 99 mL sterile BPD.

(*2*) *All other foods*.—Weigh a 50 g test portion into a sterile stomacher bag and dilute with 450 mL BPD; blend or homogenize per standard.

(b) Prepare 10-fold serial dilutions in BPD.

(c) Place two 3M Petrifilm RAC Plates on a flat, level surface for each dilution to be tested.

(d) Lift the film. With the pipet perpendicular, dispense 1 mL of each dilution onto the center of the bottom film of each plate.

(e) Roll the film down onto the sample.

(f) Place the 3M Petrifilm Flat Spreader on the center of the plate. Press gently on the center of the spreader to distribute the sample evenly. Spread the inoculum over the entire 3M Petrifilm RAC Plate growth area before the gel is formed. *Do not slide the spreader across the film*.

(g) Remove the spreader and leave the plate undisturbed for at least one minute to permit the gel to form

(h) Incubate the 3M Petrifilm RAC Plates at either 32 ± 1°C (seafood and dairy products) or 35 ± 1°C (all other foods) in a horizontal position with the clear side up in stacks of no more than

Table 2015.13D. Log_{10} total aerobic bacterial counts for raw easy-peel shrimp by 3M Petrifilm RAC Plate incubated at 32°C vs FDA BAM Chapter 3 reference method

	Low				Medium				High			
	Petrifilm RAC[a]		FDA BAM[b]		Petrifilm RAC		FDA BAM		Petrifilm RAC		FDA BAM	
	Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g	
Laboratory	A[c]	B	A	B	A	B	A	B	A	B	A	B
1	3.000	3.065	3.182	3.034	4.389	4.785	4.159	4.231	5.344	5.558	5.852	6.000
2	2.864	2.858	3.423	3.121	4.288	4.238	4.057	4.306	5.344	5.207	5.881	5.749
3	3.176	2.950	3.297	3.297	4.210	4.373	4.279	4.366	5.558	5.479	6.072	6.017
4	2.964	3.000	3.686	3.398	4.156	4.292	4.423	4.447	5.664	5.479	6.165	6.118
5	2.779	2.717	3.191	3.202	4.114	4.072	4.251	4.303	5.258	5.004	5.870	5.749
6	3.236	3.153	2.644	2.846	3.764[d]	4.623	4.000	3.955	5.004	5.281	5.654	5.717
7	3.179	3.127	2.634	2.344	4.009	4.477	3.974	3.708	5.603	5.644	5.757	5.364[d]
8	2.700	2.786	2.364	2.654	3.969	3.925	3.881	3.682	5.344	5.382	5.479	5.581
9	3.114	3.490	3.191	3.505	4.403	4.366	4.270	4.299	5.827	5.833	5.909	5.974
10	3.176	3.212	2.479[d]	3.369	4.439	4.439	4.407	4.415	5.914	6.005	5.925	6.087
11	2.876	2.876	3.134	3.094	4.388	4.108	4.000	3.846	5.664	5.893	5.925	5.820
12	2.624	2.807	2.417	2.793	4.199	4.127	3.772	3.974	5.624	5.581	5.533	5.417
13	3.050	3.042	3.121	3.388	4.083	4.193	4.492	4.312	5.507	5.664	5.682	5.644
14	2.479[d]	3.026	3.251	3.260	4.316	4.412	4.377	4.418	5.833	5.624	6.065	5.969
15	2.303	2.258	3.162	3.097	4.455	4.512	3.400	3.417	5.682	5.749	5.644	5.654
16	3.246	3.256	3.322	3.343	4.057	4.226	4.176	4.207	5.493	5.558	5.992	5.904

[a] RAC = 3M Petrifilm Rapid Aerobic Count Plate method.

[b] FDA BAM = FDA BAM Chapter 3 (Aerobic Plate Count) method.

[c] A and B are the indicated duplicate test portions.

[d] Cochran's outlier.

Table 2015.13E. Log_{10} total aerobic bacterial counts for raw easy-peel shrimp by 3M Petrifilm RAC Plate incubated at 35°C vs FDA BAM Chapter 3 reference method

	Low				Medium				High			
	Petrifilm RAC[a]		FDA BAM[b]		Petrifilm RAC		FDA BAM		Petrifilm RAC		FDA BAM	
	Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g	
Laboratory	A[c]	B	A	B	A	B	A	B	A	B	A	B
1	2.558	2.757	3.182	3.034	4.146	4.118	4.159	4.231	5.433	5.846	5.852	6.000
2	2.682	2.772	3.423	3.121	4.306	4.188	4.057	4.306	5.507	5.464	5.881	5.749
3	3.042	2.876	3.297	3.297	4.193	4.308	4.279	4.366	5.793	5.793	6.072	6.017
4	2.814	2.935	3.686	3.398	4.127	4.246	4.423	4.447	5.614	5.603	6.165	6.118
5	2.741	2.717	3.191	3.202	4.118	4.042	4.251	4.303	5.592	5.344	5.870	5.749
6	3.329	3.341	2.644	2.846	3.207[d]	3.983	4.000	3.955	5.807	5.581	5.654	5.717
7	2.700	2.507	2.634	2.344	4.320	4.114	3.974	3.708	5.800	5.925	5.757	5.364[d]
8	2.045	2.382	2.364	2.654	3.914	3.749	3.881	3.682	5.344	5.382	5.479	5.581
9	2.700	3.072	3.191	3.505	4.403	4.366	4.270	4.299	5.644	5.717	5.909	5.974
10	2.846	2.893	2.479[d]	3.369	4.362	4.243	4.407	4.415	5.820	5.909	5.925	6.087
11	2.717	2.700	3.134	3.094	4.322	4.034	4.000	3.846	5.581	5.950	5.925	5.820
12	2.083	2.149	2.417	2.793	3.839	4.050	3.772	3.974	5.793	5.382	5.533	5.417
13	2.664	2.904	3.121	3.388	4.379	4.127	4.492	4.312	5.533	5.603	5.682	5.644
14	2.749	2.717	3.251	3.260	4.087	4.193	4.377	4.418	5.800	5.708	6.065	5.969
15	3.343	3.279	3.162	3.097	3.533	3.533	3.400	3.417	5.717	5.749	5.644	5.654
16	3.173	3.042	3.322	3.343	4.080	4.193	4.176	4.207	5.717	5.717	5.992	5.904

[a] RAC = 3M Petrifilm Rapid Aerobic Count Plate method.

[b] FDA BAM = FDA BAM Chapter 3 (Aerobic Plate Count) method.

[c] A and B indicate duplicate test portions.

[d] Cochran's outlier.

Table 2015.13F. Log_{10} total aerobic bacterial counts for pasteurized skim milk by 3M Petrifilm RAC Plate vs SMEDP Chapter 6 reference method

	Low				Medium				High			
	Petrifilm RAC[a]		SMEDP[b]		Petrifilm RAC		SMEDP		Petrifilm RAC		SMEDP	
	Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g	
Laboratory	A[c]	B	A	B	A	B	A	B	A	B	A	B
1	2.303	2.303	2.324	2.004	3.324	3.507	3.258	3.558	4.569	4.581	4.449	4.449
2	3.146	2.904	3.188	2.893	3.904	4.013	3.800	3.914	4.644	4.592	4.708	4.603
3	2.644	2.324	2.733	2.417	3.382	3.793	3.149	3.779	4.757	4.820	4.682	4.807
4	2.507	2.364	2.569	2.433	3.382	3.507	3.344	3.603	4.558	4.682	4.624	4.644
5	2.749	2.945	2.725	2.852	3.708	3.741	3.614	3.581	4.382[d]	4.904	4.303[d]	4.779
6[e]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7[e]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
8[e]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	2.507	2.533	2.417	2.382	3.700	3.558	3.581	3.449	4.864	4.614	4.764	4.479
10	2.592	2.533	2.449	2.382	3.624	3.592	3.149	3.433	4.654	4.682	4.581	4.654
11	2.581	2.149	2.417	2.233	3.207	3.479	3.233	3.464	4.558	4.417	4.507	4.433
12	2.741	2.764	2.725	2.558	3.507	3.624	3.207	3.433	5.080	4.846	4.717	4.700
13	2.207	2.207	2.083	2.258	3.233	3.179	3.258	3.149	4.281	4.449	4.344	4.382
14	2.233	2.149	2.149	2.233	3.149	3.179	3.149	3.303	4.382	4.303	4.344	4.382
15	1.908	2.083	2.083	2.045	3.258	3.207	3.382	3.507	4.382	4.303	4.258	4.303
16	2.793	2.793	2.708	2.733	3.964	3.814	3.893	3.786	4.955	5.022	4.914	4.920

[a] RAC = 3M Petrifilm Rapid Aerobic Count Plate method.

[b] SMEDP = Standard Methods for the Examination of Dairy Products Chapter 6 (Standard Plate Count Method).

[c] A and B indicate duplicate test portions.

[d] Cochran's outlier.

[e] NA = Not available, laboratory did not participate, or did not submit data.

Table 2015.13G. Log_{10} total aerobic bacterial counts for instant NFDM by 3M Petrifilm RAC Plate vs SMEDP Chapter 6 reference method

	Low				Medium				High			
	Petrifilm RAC[a]		SMEDP[b]		Petrifilm RAC		SMEDP		Petrifilm RAC		SMEDP	
	Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g		Log_{10} CFU/g	
Laboratory	A[c]	B	A	B	A	B	A	B	A	B	A	B
1	2.400	2.449	2.083	2.364	3.034	3.087	2.800	2.820	4.391	4.243	3.955	3.858
2	2.364	2.449	2.258	2.344	2.974	2.992	2.779	2.793	4.215	4.165	4.199	4.231
3	2.149	2.233	2.303	2.258	2.935	2.914	3.009	2.974	4.188	4.375	4.108	4.292
4	2.479	2.083[d]	2.417	2.083[d]	2.876	2.779	2.700	2.664	4.210	4.623	4.140	4.720
5	2.382	2.417	2.417	2.464	3.005	3.087	2.820	2.935	4.246	4.162	4.199	4.137
6	2.417	2.507	2.083	2.400	3.290	3.371	3.146	3.312	4.322	4.241	4.256	4.061
7	2.417	2.382	2.149	2.149	2.881	3.034	2.950	2.870	4.215	4.146	4.199	4.168
8[e]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	2.479	2.449	2.417	2.533	2.978	3.053	2.898	2.945	4.046	4.256	4.026	4.362
10	2.400	2.479	2.258	2.449	3.000	3.000	2.904	3.050	4.000	4.000	3.807	3.992
11	2.581	2.533	2.464	2.569	3.173	3.140	3.114	3.171	4.295	4.193	4.279	4.215
12	2.533	2.558	2.258	2.004[d]	3.061	2.992	3.231	3.080	4.108	4.368	4.000	4.299
13	2.433	2.400	2.149	2.207	3.026	3.080	2.945	2.914	4.156	4.716	4.134	4.681
14	2.479	2.581	2.624	2.644	3.140	3.159	2.955	2.881	4.065	4.398	4.094	4.306
15	2.382	2.520	2.644	2.449	3.407	3.204	3.613[d]	3.143	4.484	4.591	4.267	4.176
16	2.344	2.117	2.382	2.207	2.852	2.876	2.983	2.964	4.162	3.846	4.065	3.974

[a] RAC = 3M Petrifilm Rapid Aerobic Count Plate method.

[b] SMEDP = Standard Methods for the Examination of Dairy Products Chapter 6 (Standard Plate Count Method).

[c] A and B indicate duplicate test portions.

[d] Cochran's outlier.

[e] NA = Not available, laboratory did not participate, or did not submit data.

20 (dairy products) or 40 for all other foods. Enumerate plates after 24 ± 2 h of incubation (or 48 ± 3 h in the case of dairy powders, including whey powder). 3M Petrifilm RAC Plates can be counted using a standard colony counter with the use of a back-light or an illuminated magnifier to assist with the estimated enumeration.

(i) Enumerate all colonies regardless of size, color, or intensity.

(j) The circular growth area is approximately 30 cm^2. Plates containing >300 colonies can be either estimated or recorded as TNTC. Estimation can only be done by counting the number of colonies in one or more representative squares and determining the average number per square. The average number can be multiplied by 30 to determine the estimated count per plate. If a more accurate count is required, the sample may need to be retested at higher dilutions.

(k) Average the counts between the replicate plates. Report final results as CFU per gram or milliliter (CFU/g or CFU/mL). *Note*: If there are two dilutions within the countable range, use the following calculation to determine the final count:

$$N = \Sigma C / \left(1.1 \times d\right)$$

where, N is the number of colonies per milliliter or per gram of product, ΣC is the sum of all colonies on both plates, and *d* is the dilution from which first counts were obtained.

(l) Food samples may occasionally show interference on the 3M Petrifilm RAC Plates for example: (*1*) Uniform blue background color (often seen from the organisms used in cultured products). These should not be counted as TNTC.

(*2*) Intense pinpoint blue specs (often seen with spices or granulated products).

(m) When necessary, colonies may be isolated for further identification test using standard procedures. Lift the top film and pick the colony from the gel.

Results of the Collaborative Study

In this collaborative study, the 3M Petrifilm RAC Plate was compared with two reference methods for the enumeration of aerobic bacteria: FDA BAM Chapter 3 (for raw easy-peel shrimp) and SMEDP Chapter 6 (for instant NFDM). A total of 16 laboratories throughout the United States participated in the evaluation (all 16 laboratories participated in the evaluation of the raw easy-peel shrimp, with 15 laboratories participating in the evaluation of the pasteurized skim milk and instant NFDM) with 16 laboratories submitting data for raw easy-peel shrimp, 13 laboratories submitting data for the pasteurized skim milk, and 15 laboratories submitting data for the instant NFDM as presented in Table **2015.13C**. For the raw easy-peel shrimp and dry milk power, all participating laboratories submitted data. For pasteurized skim milk, two laboratories reported deviations from the protocol and their data were not included in the statistical analysis. After receipt of samples, Laboratory 6 indicated that samples were stored at room temperature (24 ± 2°C) instead of refrigeration temperature (2–8°C) for 24 h prior to testing. Laboratory 7 reported that their samples were not received until after 48 h of shipment. The temperature control indicated the samples were at extremely elevated temperature (30°C). Both laboratories proceeded with sample analysis, however all samples analyzed produced results that were greater than the countable range (TNTC) for all dilutions and no data were submitted.

The 3M Petrifilm RAC Plate results along with FDA BAM and SMEDP results reported by each laboratory were converted to logarithmic values for statistical analysis and plotted using a Youden plot. The Log_{10} individual laboratory results are presented in Tables **2015.13D-G**. Figures 1–4 present the Youden plots for each method for each matrix. Figures 5–8 present the mean Youden plots for 3M Petrifilm RAC and the reference methods. The transformed data were analyzed for outliers by Cochran and Grubbs' tests. No evidence of physical cause or suspicion of cause was noted, so all identified outliers were included in the statistical analysis. The difference of means and the reverse-transformed difference of means (including 95% CIs) were determined for each contamination level for each matrix to determine whether a statistically significant difference existed between the methods. s_r and s_R were determined for each contamination level for both the 3M Petrifilm RAC Plate and FDA BAM and SMEDP methods. The results of the

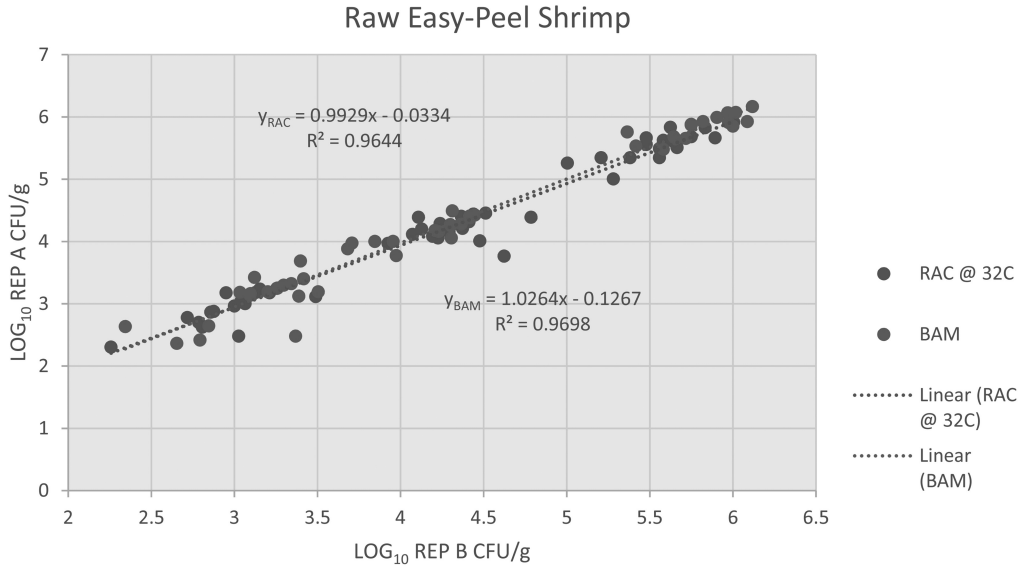

Figure 1. Youden plots for 3M Petrifilm RAC Plate and FDA BAM results for raw easy-peel shrimp evaluated at 32°C.

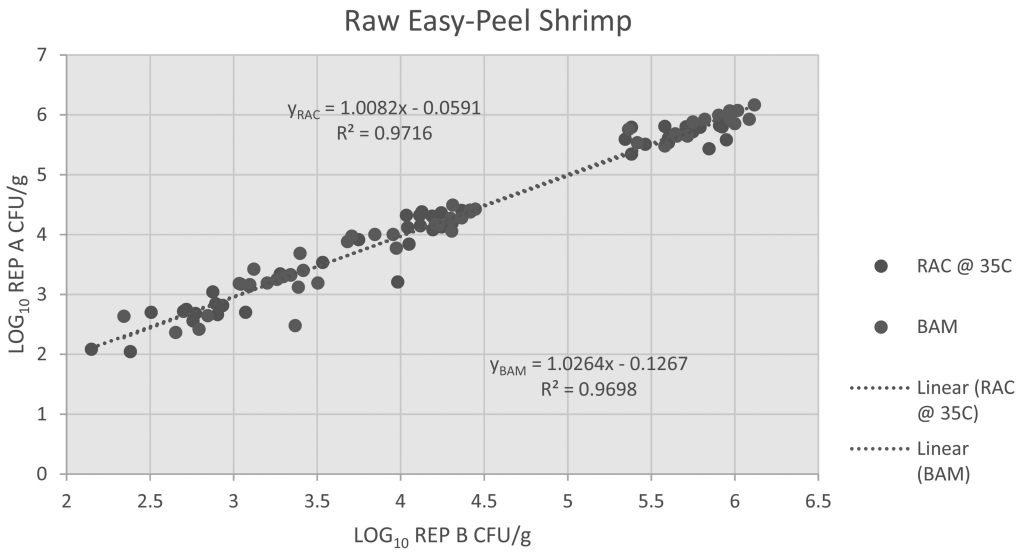

Figure 2. Youden plots for 3M Petrifilm RAC Plate and FDA BAM results for raw easy-peel shrimp evaluated at 35°C.

interlaboratory data analyses are presented in Tables **2015.13A** and **2015.13B**.

Raw Easy-Peel Shrimp

Raw easy-peel shrimp test portions were evaluated at a low, medium, and high contamination level for the presence of naturally occurring aerobic bacteria. Replicate 3M Petrifilm RAC Plates were evaluated at both 32 and 35°C (Tables **2015. 13D and E**) Sixteen laboratories participated in the analysis of this matrix, with all 16 laboratories submitting data.

3M Petrifilm RAC Plate Incubated at 32°C

For the 3M Petrifilm RAC Plate low and medium contamination levels and for the FDA BAM low and high contamination levels, one outlier laboratory was identified by the Cochran's tests for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that the data would be included in the statistical analysis.

There were no statistically significant differences between the 3M Petrifilm RAC Plate and FDA BAM methods as determined by the 95% CI of the differences of means at any of the three contamination levels.

3M Petrifilm RAC Plate Incubated at 35°C

For the 3M Petrifilm RAC Plate medium contamination level and for the FDA BAM low and high contamination levels, one outlier laboratory was identified by the Cochran's tests for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that the data would be included in the statistical analysis.

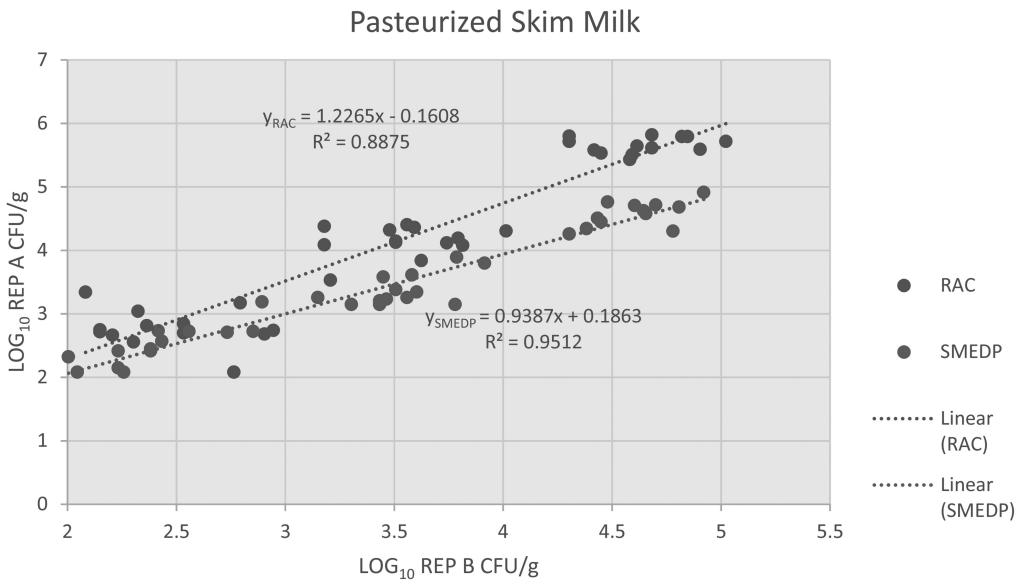

Figure 2. Youden plots for 3M Petrifilm RAC Plate and SMEDP results for pasteurized skim milk.

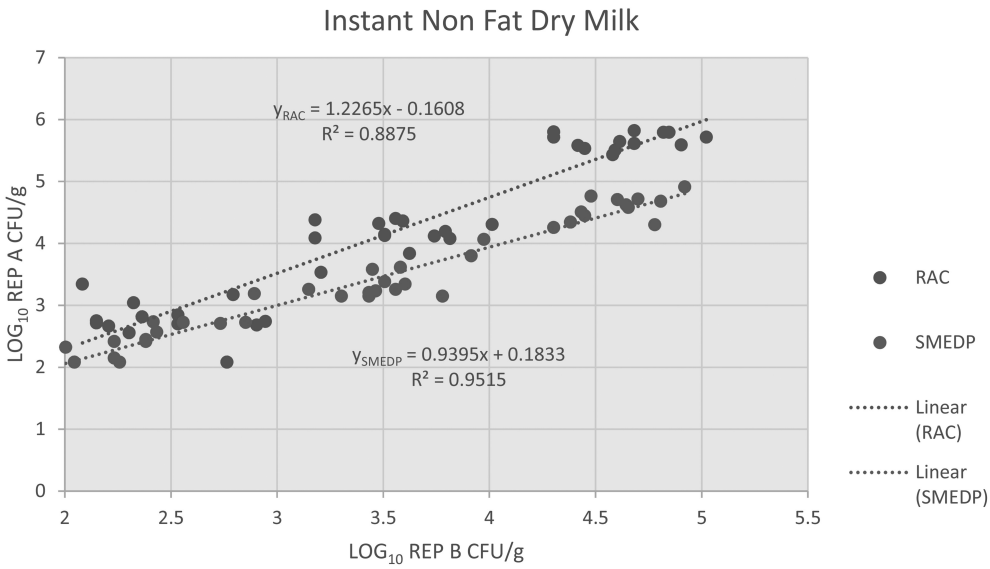

Figure 3. Youden plots for 3M Petrifilm RAC Plate and SMEDP results for instant NFDM.

There were no statistically significant differences between the 3M Petrifilm RAC Plate and FDA BAM methods as determined by the 95% CI of the differences of means at any of the three contamination levels.

Pasteurized Skim Milk

Pasteurized skim milk test portions were evaluated at a low, medium, and high contamination level for the presence of naturally occurring aerobic bacteria. Fifteen laboratories participated in the analysis of this matrix, with 13 laboratories submitting data.

For the SMEDP high contamination level, one outlier laboratory was identified by the Cochran's tests for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that the data would be included in the statistical analysis.

There were no statistically significant differences between the 3M Petrifilm RAC Plate and SMEDP methods as determined by the 95% CI of the differences of means at any of the three contamination levels.

Instant NFDM

Instant NFDM test portions were evaluated at a low, medium, and high contamination level for the presence of naturally occurring aerobic bacteria. Fifteen laboratories participated in the analysis of this matrix, with 15 laboratories submitting data.

For the 3M Petrifilm RAC Plate low contamination level and for the SMEDP medium contamination level, one outlier laboratory was identified by the Cochran's tests for outliers.

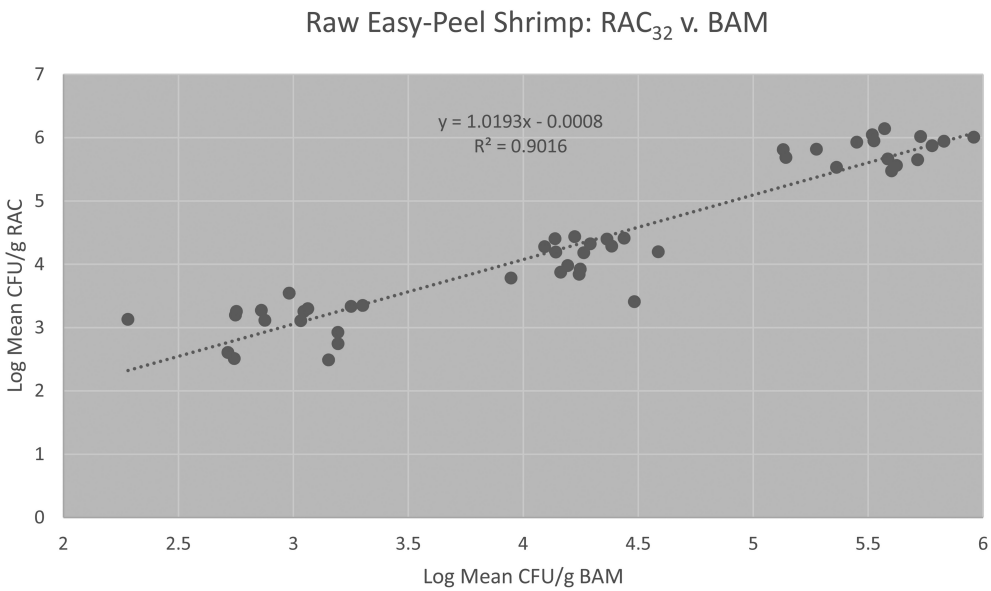

Figure 5. Youden plot for combined mean 3M Petrifilm RAC Plate and FDA BAM results for raw easy-peel shrimp evaluated at 32°C.

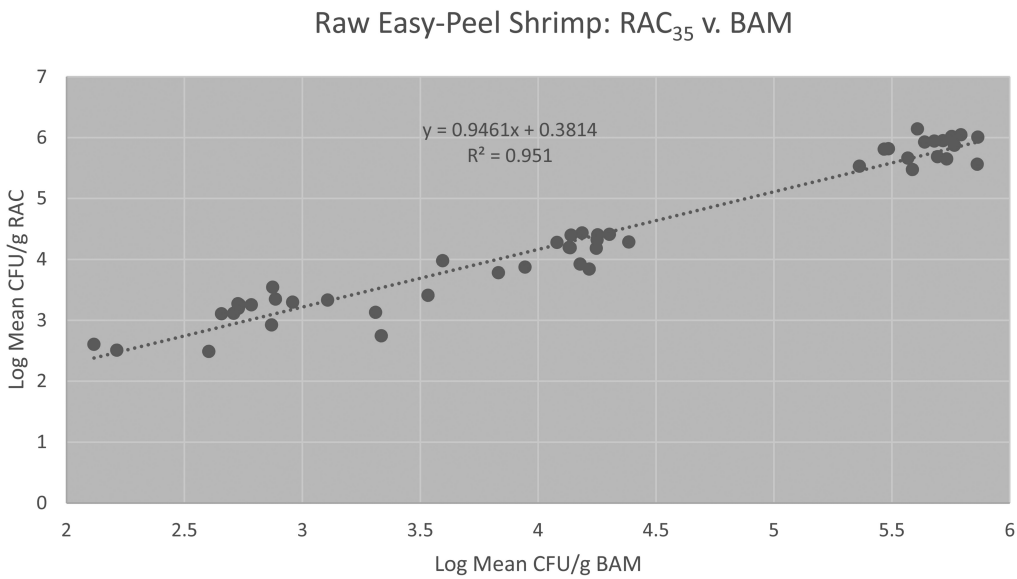

Figure 6. Youden plot for combined mean 3M Petrifilm RAC Plate and FDA BAM results for raw easy-peel shrimp evaluated at 35°C.

However, no evidence of physical cause or suspicion of cause was noted and it was determined that the data would be included in the statistical analysis.

There were no statistically significant differences between the 3M Petrifilm RAC Plate and SMEDP methods as determined by the 95% CI of the differences of means at any of the three contamination levels.

Discussion

No negative feedback was reported to the study directors from the collaborating laboratories in regards to the performance of the 3M Petrifilm RAC Plate. Several laboratories indicated that the colonies were more easily identified on the 3M Petrifilm RAC plates then on the reference method agar plates due to "vibrant colony color and intensity" observed during the evaluation. For the instant NFDM, several laboratories indicated the 3M Petrifilm RAC plates prevented colonies from producing spreader colonies, which had occurred on the reference method agar plates. This allowed for easier enumeration on the 3M Petrifilm RAC plates than the reference method agar plates. Additionally, one laboratory indicated, "For laboratories working with dairy products or with products that contain high levels of *Bacillus*, the RAC plates would provide a significant benefit."

During the analysis of the pasteurized skim milk, two laboratories indicated deviations from the approved protocol and did not submit data for statistical analysis. Laboratory 7 received their test portions after 48 h from the initial shipment from the coordinating laboratory. The temperature control indicated that the samples were outside the acceptable range, however, the

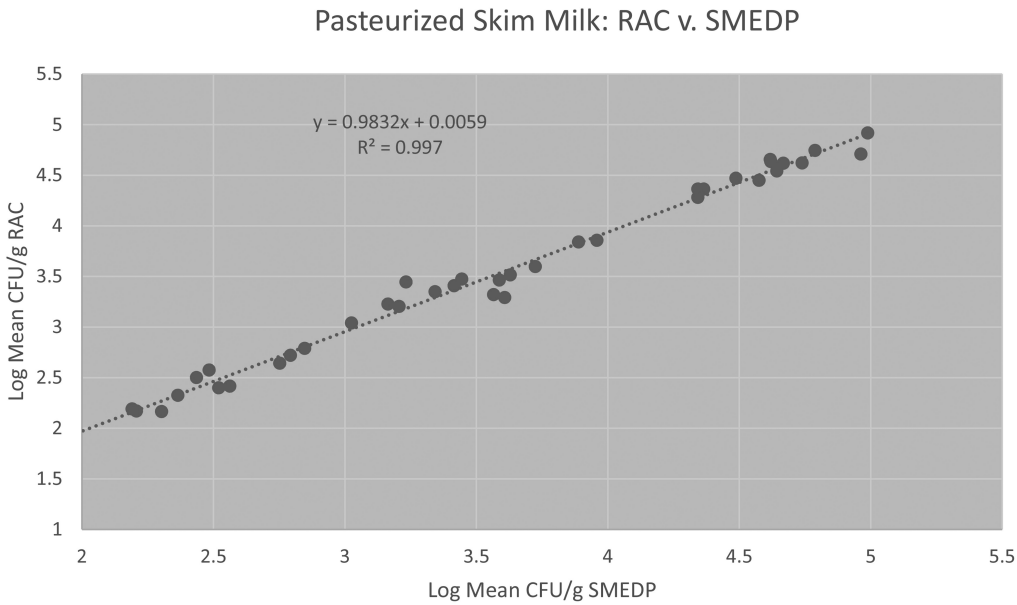

Figure 7. Youden plot for combined mean 3M Petrifilm RAC Plate and SMEDP results for pasteurized skim milk.

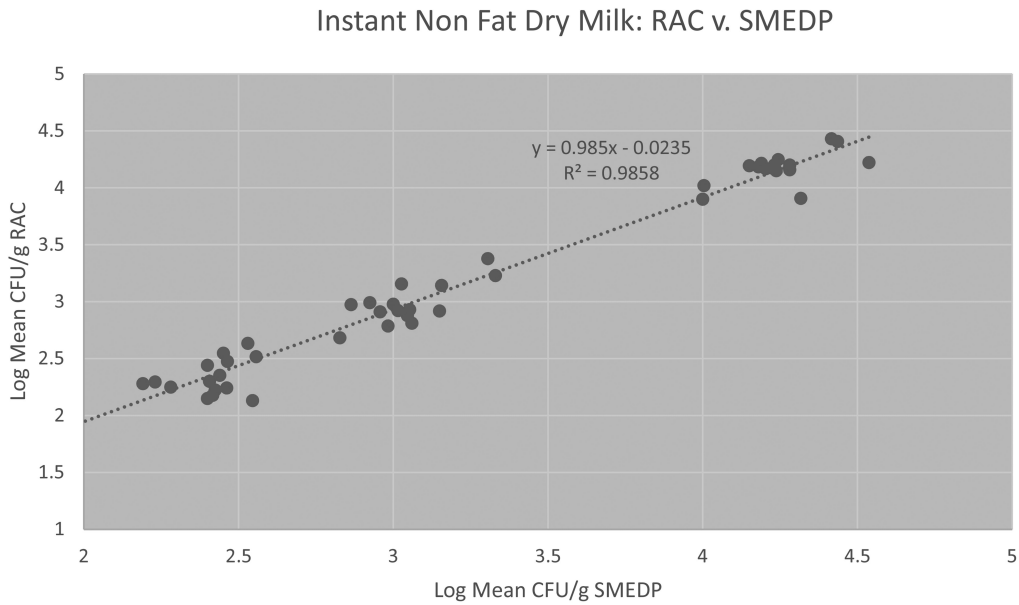

Figure 8. Youden plot for combined mean 3M Petrifilm RAC Plate and SMEDP results for instant NFDM.

laboratory did continue analysis of the test portions. For each sample, TNTC results were obtained on all dilutions prepared for both the 3M Petrifilm RAC plate and the SMEDP reference method agar plates. The coordinating laboratory was notified of the shipment error and no additional data were submitted for the matrix. Laboratory 6 indicated to the coordinating laboratory that their sample test portions were stored at room temperature (24 ± 2°C) upon receipt for 24 h prior to analysis. For each sample, TNTC results were obtained on all dilutions prepared for both the 3M Petrifilm RAC plate and the SMEDP reference method agar plates. The coordinating laboratory was notified of the error and no additional data were submitted for the matrix.

No statistically significant difference was observed between the 3M Petrifilm RAC Plate and FDA BAM or SMEDP reference methods for all three matrixes evaluated when compared using the difference of means, including 95% CI. Based on the data presented, the reproducibility values obtained for the three matrixes for all three contamination levels were generally similar between the candidate and reference methods, indicating that both the between-laboratory variations and within-laboratory variations were consistent between the candidate and reference method. These values indicate that for reproducibility, no meaningful statistical differences were observed in the data between the candidate and reference methods when test portions were analyzed by different analysts at each laboratory or within each sample set at a given laboratory.

Recommendations

It is recommended that the 3M Petrifilm RAC Plate be adopted as Official First Action status for the enumeration of aerobic bacteria from raw ground beef, raw ground pork, raw ground turkey, chicken carcass rinsate, fresh swai, fresh tuna, fresh tiger shrimp, raw easy-peel shrimp, cherry tomato wash, frozen blueberries, Mediterranean apricots, creamy salad dressing, fresh pasta, vanilla ice cream, instant NFDM, and pasteurized skim milk.

Acknowledgments

We extend a sincere thank you to the following collaborators for their dedicated participation in this study:

Leslie Thompson (Vanguard Sciences, North Sioux City, ND)

Heidi Wright (AEMTEK Inc., Freemont, CA)

Luci Hardrath (AGSOURCE Laboratories, Marshfield, WI)

Wendy McMahon, Brian Kupski, and Nicole Cuthbert (Silliker Food Science Center, Crete, IL)

Joe Meyer and Jean Schoeni (Covance Laboratories, Madison, WI)

Karilyn Gonzales and Chelsea Fagundes (Food Microbiological, Cypress, CA)

Chris Lopez, Alex Brandt, and Bharath Brahmanda (Food Safety Net Services, San Antonio, TX)

Joey Marchant-Tambone (FDA. CFSAN Gulf Coast Seafood Laboratory, Dauphin Island, AL)

Adam Hankins (McCoy & McCoy Laboratories, Inc., Madisonville, KY)

Ashley Morris and Robert Brooks (Microbac Laboratories, Inc., Maryville, TN)

Karen Beers (Safe Foods/MCA, Rogers, AR)

Jerri Lynn Pickett, Joseph Reynolds, and Kierra Payne-McDaniel (Tyson-WBA Analytical, Springdale, AR)

Dora Rodgers and Kate Angeles (Wisconsin Department of Agriculture, Trade and Consumer Protection Bureau of Laboratory Services, Madison, WI)

Brenda Brown (Murray Brown Laboratories LLC, Madison, WI)

Kevin Miller (Marshfield Food Safety, LaCrosse, WI)

Allison Mastalerz (Q Laboratories, Inc., Cincinnati, OH)

We also extend special thanks to the following team members at Q Laboratories, Inc. for their efforts during the collaborative study: M. Joseph Benzinger, Jr., Kiel Fisher, Megan Boyle, Ben Bastin, Alison DeShields, Emmanuel Onuoha, T. Shane Wilson, Erica Lee, and Nicole Klass.

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AOAC Official Method 2015.14
Simultaneous Determination of Total Vitamins B_1, B_2, and B_6
in Infant Formula and Related Nutritionals
Enzymatic Digestion and LC-MS/MS
First Action 2015

A. Principle

This method facilitates simultaneous quantitation of four water-soluble vitamins (WSV) in infant formula and related nutritional products, including all SPIFAN SMPR relevant forms of vitamins B_1, B_2, B_3, and B_6. Samples are prepared by enzymatic digestion with papain and α-amylase to hydrolyze protein and complex carbohydrate and acid phosphatase to free phosphorylated vitamin forms. Stable-isotope labeled internal standards are incorporated into the sample preparation to correct for variability in both the sample preparation and instrument response. A series of six mixed working standard solutions spanning two orders of magnitude in vitamin concentration are used to generate calibration curves based on the peak response ratio of the analyte to its stable-isotope labeled internal standard.

Prepared samples and working standard solutions are injected onto ultra-high pressure liquid chromatograph (UPLC) interfaced to a triple-quadrupole mass spectrometer (MS/MS) for analysis. The MS/MS is configured to monitor parent-daughter (precursor-fragment) ion pairs for each analyte and internal standard. This reaction forms the basis for method selectivity. Analytes are quantified by least squares regression using the response ratio of the analyte to its internal standard.

B. Apparatus and Materials

1. Control Sample: NIST SRM 1849a, or current lot. Store at 4°C.
2. Waters Acquity BEH C18 column, 2.1 x 100, 1.7 µm. Part# 186002352.
3. UPLC system: Waters Acquity Classic, or equivalent.
4. Tandem quadrupole mass spectrometer with ESI probe: Waters Xevo TQ-S, or equivalent.
5. Analytical balances (3):
   a) One capable of accurately weighing 5.00 mg (for standards), 6-place balance.
   b) An analytical five-place balance for samples, and
   c) A top loading two place balance capable of weighing to several hundred grams.
6. Water purifier: Millipore Milli-Q Water Purification System, or equivalent.
7. Water bath shaker capable of maintaining 37°C, Lab-Line Orbit, or equivalent.
8. Bottle-top dispenser: capable of dispensing volumes of approximately 24 mL.
9. pH meter: capable of measuring a pH of 4.0 - 5.0.
10. Vortex mixer.
11. Multi-position magnetic stir plate.

12. Room Light Shields: A.L.P. Protect-A-Lamp, UV cutoff at 460 nm, or equivalent.
13. Graduated cylinders: various sizes, including: 10, 100, 500, and 1000 mL.
14. Beakers: various sizes, including: 100, 200, 400, 600, 1000, and 2000 mL.
15. Volumetric flasks: various sizes, including: 10, 25, 50, 100, 250, and 2000 mL.
16. Mobile phase bottles: glass, various sizes, including: 250, 500, 1000, and 2000 mL .
17. Disposable, plastic Pasteur pipettes.
18. Amber bottles: volume capacity of 50 and 100 mL (for stock standard storage).
19. Weighing vessels: various, including disposable weighing boats and glass weighing funnels.
20. Positive displacement pipettes: Gilson Microman - 10, 100, 250, and 1000 µL; Part #'s F148501, F148504, F148505, and F148506.
21. Positive displacement pipette tips: Gilson Capillary Piston - 10, 100, 250, and 1000 µL; Part #'s F148312, F148314, F148014, and F148560.
22. Plastic syringes, 3 mL.
23. Syringe filters, PTFE 0.45 µm syringe filters: Acrodisc 25 mm, or equivalent.
24. 50 mL self-standing, plastic centrifuge tubes: Superior Scientific, Ltd., or equivalent.
25. Autosampler vials: Waters autosampler vials; 9 mm amber with screw top 12x32 mm pre-split PTFE-silicon septa; Waters Part # 186000847C, or equivalent.
26. Teflon coated magnetic stir bars.

C. Reagents

1. Nicotinamide: USP Reference Standard (U.S.P.C., Inc. Rockville, MD) Official Lot; Catalog # 1462006. Store as indicated on label.
2. Niacin (nicotinic acid): USP Reference Standard (U.S.P.C., Inc. Rockville, MD) Official Lot; Catalog # 1461003. Stored as indicated on label.
3. Pyridoxine hydrochloride: USP Reference Standard (U.S.P.C., Inc. Rockville, MD) Official Lot; Catalog # 1587001. Store in desiccator protected from white light. Dry according to manufacturer's instructions prior to use.
4. Riboflavin: USP Reference Standard (U.S.P.C., Inc. Rockville, MD) Official Lot; Catalog # 1603006. Store in desiccator protected from white light. Dry according to manufacturer's instructions prior to use.
5. Thiamine hydrochloride: USP Reference Standard (U.S.P.C., Inc. Rockville, MD) Official Lot; Catalog # 1656002. Store in desiccator protected from white light. Measure the moisture content of the powder prior to use.
6. Pyridoxamine dihydrochloride: Fluka Analytical Standard, catalog# P9380.
7. Pyridoxal hydrochloride: Sigma, catalog# P9130.
8. $^{2}H_{4}$-Nicotinamide: CDN Isotopes; Catalog # D-3457.
9. $^{2}H_{4}$-Nicotinic acid: CDN Isotopes; Catalog # D-4368.

10. $^{13}C_4$-Pyridoxine: pyridoxine:HCl (4,5-bis(hydroxymethyl)-$^{13}C_4$); Cambridge Isotope Laboratory; Catalog # CLM-7563.
11. $^{2}H_3$-Pyridoxal: IsoSciences; Catalog # 7098.
12. $^{2}H_3$-Pyridoxamine: IsoSciences; Catalog # 7099.
13. $^{13}C_4$-Thiamine chloride; IsoSciences; Catalog # 9209.
14. $^{13}C_4$,$^{15}N_2$-Riboflavin: IsoSciences, Catalog# 7072.
15. Acid phosphatase, type II from potato, 0.5 – 3.0 U/mg: Sigma, catalog# P3752.
16. Papain from Carica papaya, ≥ 3 U/mg: Sigma, catalog# 76220.
17. α–amylase from aspergillus oryzae, 150 U/mg: Sigma, catalog# A9857.
18. Hydrochloric acid concentrated (12M); ACS grade, or equivalent.
19. Ammonium formate: for mass spectrometry (≥99.0%), Fluka 70221 or equivalent.
20. Glacial acetic acid: Sigma ACS Reagent Grade, or equivalent.
21. Formic acid: Sigma ACS Reagent Grade, or equivalent.
22. Laboratory Water: 18.0 MΩ, < 10 ppb TOC, or equivalent.
23. Methanol: Fisher LC-MS/MS Optima grade or EMD Omni-Solve LC-MS grade.
24. Ethylenediaminetetracetic acid, disodium salt dihydrate (EDTA): ACS grade (99-101%), or equivalent.
25. Potassium phosphate dibasic: ACS grade (>98%), or equivalent.
26. meta-Phosphoric acid: ACS grade (33.5-36.5%), or equivalent.
27. pH 4.0, 7.0, and 10.0 buffer solutions for pH meter calibration.
28. Phosphoric acid, 85%, ACS grade, or equivalent.
29. Potassium hydroxide, 40%, ACS grade, or equivalent.

D. Standard and Solution Preparation

1. Mobile Phase A: 20 mM ammonium formate in water - Using a graduated cylinder, transfer 500 mL laboratory water to a mobile phase reservoir. Add 0.631 g of ammonium formate and mix well. Scale as needed. Expiration: 3 days.
2. Mobile Phase B: Methanol.
3. Weak needle wash: 10% methanol in water, expiration 3 mo.
4. Strong needle wash: Methanol.
5. 50 mM ammonium formate - Using a graduated cylinder, transfer 1400 mL laboratory water to an appropriate reservoir. Add 4.41 g of ammonium formate and mix well. 1400 mL is adequate for 6 working standards and 32 samples. Scale as needed. Expiration: 3 days.
6. Enzyme Cocktail - Using a graduated cylinder, transfer 200 mL 50 mM ammonium formate buffer to an appropriate reservoir. Add 200 ± 10 mg acid phosphatase, 80 ± 5 mg α-amylase,

and 400 ± 10 mg papain. Mix for ten minutes with magnetic stir plate and stir bar. Adjust to pH 4.0-4.5 with formic acid (~ 100 µL). 200 mL is adequate for 6 working standards and 32 samples. Scale as needed. Prepare fresh daily.

5. Stable-Isotope Labeled Compounds – Individual, Internal Standard Stock Solutions

   a) $^{2}H_4$-Nicotinamide Stock Solution (Approximate Concentration: 560 µg/mL) - Weigh 14.0 ± 0.1 mg into a tarred weighing vessel. Quantitatively transfer to a 25 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 50 mL amber bottle and Store refrigerated (2˚-8˚C). Expiration: Until exhausted or evidence of contamination.

   b) $^{2}H_4$-Nicotinic Acid Stock Solution (Approximate Concentration: 500 µg/mL) - Weigh 12.5 ± 0.1 mg into a tarred weighing vessel. Quantitatively transfer to a 25 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 50 mL amber bottle and Store refrigerated (2˚-8˚C). Expiration: Until exhausted or evidence of contamination.

   c) $^{13}C_4$-Pyridoxine Stock Solution (Approximate Concentration: 70 µg/mL) - Weigh 7.0 ± 0.1 mg into a tarred weighing vessel. Quantitatively transfer to a 100 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 100 mL amber bottle and Store refrigerated (2˚-8˚C). Expiration: Until exhausted or evidence of contamination.

   d) $^{2}H_3$-Pyridoxal Stock Solution (Approximate Concentration: 40 µg/mL) - Weigh 4.0 ± 0.1 mg into a tarred weighing vessel. Quantitatively transfer to a 100 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 100 mL amber bottle and Store refrigerated (2˚-8˚C). Expiration: Until exhausted or evidence of contamination.

   e) $^{2}H_3$-Pyridoxamine Stock Solution (Approximate Concentration: 40 µg/mL) - Weigh 4.0 ± 0.1 mg into a tarred weighing vessel. Quantitatively transfer to a 100 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 100 mL amber bottle and Store refrigerated (2˚-8˚C). Expiration: Until exhausted or evidence of contamination.

   e) $^{13}C_4$-Thiamine Chloride Stock Solution (Approximate Concentration: 100 µg/mL)

      i) 0.12 M HCl - Add approximately 300 mL water to a 500 mL graduated cylinder. Add 5.0 ± 0.1 mL conc. HCl and swirl to mix. Bring to 500 mL with laboratory water and mix well.

      ii) Weigh 5.0 ± 0.1 mg $^{13}C_4$-thiamine into a tarred weighing vessel. Quantitatively transfer to a 50 mL volumetric flask with 0.12 N HCl and QS with 0.12 N HCl. Mix well and transfer to a 100 mL amber bottle and Store refrigerated (2˚-8˚C). Expiration: Until exhausted or evidence of contamination.

   f) $^{13}C_4$, $^{15}N_2$-Riboflavin Stock Solution (Approximate Concentration: 73µg/mL)

      i) 1.0% Acetic acid in water - Add approximately 30 mL water to a 500 mL graduate cylinder. Add 5.0 ± 0.1 mL glacial acetic acid and swirl to mix. Bring to 500 mL with laboratory water and mix well.

ii) Weigh 7.3 ± 0.1 mg $^{13}C_4$,$^{15}N_2$-riboflavin into a tarred weighing vessel. Quantitatively transfer to a 100 mL volumetric flask with 1.0% acetic acid and QS with 1.0% acetic acid. Mix well and transfer to a 100 mL amber bottle and Store refrigerated (2˚-8˚C). Expiration: Until exhausted or evidence of contamination.

6. Internal Standard Stock Mixture (ISSM) – Combine 2500 µL of 50 mM ammonium formate with 250 µL $^{2}H_4$-nicotinamide stock, 250 µL $^{2}H_4$-nicotinic acid stock, 250 µL $^{13}C_4$-pyridoxine stock, 200 µL $^{2}H_3$-pyridoxal stock, 50 µL $^{2}H_3$-pyridoxamine stock, 250 µL $^{13}C_4$-thiamine stock, and 250 µL $^{13}C_4$,$^{15}N_2$-riboflavin acid stock. Volume provides sufficient ISSM for 6 working standards and 32 samples. Scale as needed. Prepare fresh daily.

7. Phosphate Buffer Solution, pH 5.0 (0.10M Potassium Phosphate Dibasic, 1% EDTA, 2% Metaphosphoric Acid).

a) Weigh 20.0 ± 0.2 g of EDTA into a tarred weighing vessel and quantitatively transfer to a 2000 mL beaker containing approximately 1800 mL laboratory water and add a magnetic stir bar.

b) Weigh 34.8 ± 0.1 g of potassium phosphate dibasic into a tarred weighing vessel and quantitatively transfer to the 2000 mL beaker already containing approximately 1800 mL laboratory water and EDTA. Mix by stirring on a magnetic stir plate until both the EDTA and potassium phosphate dibasic is completely dissolved.

c) Weigh 40.0 ± 0.2 g grams of metaphosphoric acid into a tarred weighing vessel and quantitatively transfer to the 2000 mL beaker containing approximately 1800 mL laboratory water, EDTA, and potassium phosphate dibasic. Mix by stirring on a magnetic stir plate until the metaphosphoric acid is completely dissolved.

Adjust the pH of the solution to pH 5.00 ± 0.02 using 40% potassium hydroxide or 85% phosphoric acid. Quantitatively transfer the solution to a 2000 mL volumetric flask and dilute to volume with laboratory water. Expiration: 48 hours.

8. Native Compounds – Stock Standard Solutions

a) Vitamin Standard Stock Mixture (VSSM) - Accurately weigh the indicated amounts for the following standards using separate weighing funnels or other appropriate weighing vessel and quantitatively transfer to a 100 mL volumetric flask using phosphate buffer (pH 5):

(a) Niacinamide: 70.5 ± 0.5 mg

(b) Thiamine hydrochloride: 10.5 ± 0.2 mg

Determine the moisture of the USP thiamine hydrochloride reference standard as directed on the container immediately prior to weighing. The percent moisture determined for the reference standard is used to calculate the concentration of thiamine in the VSSM.

(c) Riboflavin: 7.0 ± 0.2 mg - Dry an appropriate amount of the USP riboflavin reference standard at 105 (±1)*°C* for 2 hours (± 10 minutes) prior to weighing.

(d) Pyridoxine Hydrochloride: 10.8 ± 0.2 mg - Dry an appropriate amount of the USP pyridoxine hydrochloride reference standard over indicating absorbent *in vacuo* for 4 hours prior to weighing.

QS to volume with phosphate buffer (pH 5) solution. Heat and slowly stir until the standards have completely dissolved (riboflavin dissolves more slowly) and the solution is clear. Do not heat the solution for more than 40 minutes and do not exceed 90°C. Store refrigerated (2˚-8˚C). Expiration: 3 months.

b) Nicotinic Acid Stock Solution (550 mg/mL) - Accurately weigh 13.7 ± 0.1 mg USP niacin (nicotinic acid, catalog 1461003) reference standard. Quantitatively transfer the nicotinic acid to a 25 mL volumetric flask. Add lab water to a total volume of about 20 mL and swirl until completely dissolved. Bring to volume with lab water. Mix well. Expiration: 3 months.

c) Pyridoxal Stock Solution (140 mg/mL) - Accurately weigh 17.0 ± 0.5 mg pyridoxal dihydrochloride standard. Quantitatively transfer to a 100 mL volumetric flask. Add lab water to a total volume of about 70 mL and swirl until completely dissolved. Bring to volume with lab water. Mix well. Expiration: 3 months.

d) Pyridoxamine Stock Solution (160 mg/mL) - Accurately weigh 23.0 ± 0.5 mg pyridoxamine hydrochloride standard. Quantitatively transfer to a 100 mL volumetric flask. Add lab water to a total volume of about 70 mL and swirl until completely dissolved. Bring to volume with lab water. Mix well. Expiration: 3 months.

9. Mixed Working Standard (MWS) - Combine 500 µL VSSM, 25 µL pyridoxamine stock, 25 µL pyridoxal stock, and 65 µL nicotinic acid stock solutions in a 10 mL volumetric flask containing approximately 5 mL of 50 mM ammonium formate. Bring to volume with 50 mM ammonium formate and mix well. Prepare fresh daily.

10. Working Standard Solution Preparation

a) WS1 – Add 20 µL MWS and 980 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

b) WS2 – Add 50 µL MWS and 950 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

c) WS3 – Add 100 µL MWS and 900 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

d) WS4 – Add 200 µL MWS and 800 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

e) WS5 – Add 500 µL MWS and 500 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

f) WS6 – Add 1000 µL MWS to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

E. Procedure

1. Sample preparation

a) For powdered products, using a tarred beaker or LDPE cup, weigh 10.0 ± 0.3 g of sample. Record the weight to at least 4 significant figures. This is the powder weight. Add room temperature laboratory water to bring the total reconstituted sample weight (to include the product weight) to 100 ± 2 g. Record the weight to at least 4 significant figures. This is

the reconstitution weight. Carefully add a stir bar so as not to splash the liquid from the beaker/cup and place it onto a stir plate. Set the stir plate to stir the sample as fast as possible without causing the sample to splatter or froth. Powder samples should stir for at least 10 minutes but not more than 30 minutes.

b) For reconstituted powders and liquid products - using a tarred, 50 mL centrifuge tube, weigh the appropriate sample amount (1.000 ± 0.100 g for infant formula, 0.500 ± 0.050 g for pediatric formulas and the NIST SRM, and 0.250 ± 0.050 g for adult nutritionals). Record the weight to 0.0001 g. This is the sample weight. Add 100 µL of the internal standard mixture (ISSM) via positive-displacement pipette. Vortex to mix.

2. Enzymatic digestion - add 5 mL of enzyme cocktail to all prepared samples and working standards. Cap and vortex immediately. Incubate at 37°C overnight with agitation in water bath shaker. Remove from water bath, and add 50 mM ammonium formate buffer to bring volume to approximately 30 mL and vortex to mix. Filter ~2 mL aliquot of the sample extract into an appropriate size vial using a 0.45 µm PTFE syringe filter. Transfer 60 µL of filtrate to an autosampler vial with 940 µL of 50 mM ammonium formate buffer. Cap and vortex. The sample is ready for analysis. Samples have been determined to be stable for at least 48 hours at room temperature.
3. LC-MS/MS Analysis
   a) UPLC Conditions - Place freshly prepared mobile phases, weak needle wash, and strong needle wash onto the LC system. Purge old solvents from the solvent lines and needle washes. Injection volume is 10 µL and column temperature is 40°C. Mobile phase flow rate is 0.350 mL/min. Hold at 99% mobile phase A and 1.0% mobile phase B for 0.50 min, then ramp to 8.0% B over 2.00 min, ramp to 90% B over the next 2.50 min, and hold at 90% B for 1.00 min. Return to 99% mobile phase A and 1.0% mobile phase B over 0.10 min and hold for 1.9 min for re-equilibration. Total gradient program is 8.00 min long.
   b) MS Tune Conditions - Clean the sample cone and MS source with 5% formic acid prior to analysis. Tune conditions can vary between instrument models and appropriate balance must be struck to achieve adequate signal for each compound. Appropriate conditions must be determined experimentally for each instrument model. On a Waters TQ-S, ionization is performed by ESI+ at 2.5 kV. Additional tune conditions include: source offset of 50 V, ion block temperature of 150°C, desolvation gas temperature of 500°C, desolvation gas flow of 800 L/hr, cone gas flow of 150 L/hr, nebulizer gas pressure of 7.00 bar, collision gas flow of 0.15 mL/min with argon. Both quadrupoles are set to unit mass resolution.
   c) Mass Transitions – Mass transitions for each vitamin and its corresponding internal standard are given in Table **2015.14**. Retention time windows are also given in the table. Like the tune parameters, these parameters may need adjusted based upon instrument model.
   d) UPLC-MS/MS Equilibration – The instrument should be held at initial conditions (with mobile phase flow on and MS at temperature) for 30 - 60 min prior to injection. Alternatively, 6-10 blank injections at the start of a sequence can be used for the same purpose.

4. Quality Control
   a) Blanks of 50 mM ammonium formate need to bracket each calibration curve to enable check for laboratory background and instrumental carryover. Background should be no more than 5% of the signal for the lowest working standard.
   b) Calibration Curve – Calibration curves are set up to bracket the sample injections. Calibration residuals (relative error from known concentration) are expected to be ≤ 20% for pyridoxal and ≤ 8% for the other vitamins. A standard injection outside of this range can be excluded with evidence of a standard preparation error in a single calibration level leading to a high or low response for all vitamins or evidence of a one-off instrumental error, such as a missed injection.
   c) Laboratory Control - NIST SRM 1849a, or current lot, serves as a control sample and should be prepared and analyzed with each sample set. The control result for each analyte must be within limits established by a control chart. By and large, the levels reported by this method are within the NIST certified range because of the minute concentration of phosphorylated vitamin forms in SRM 1849a.

F. Calculations

1. Vitamin Stock Solutions concentration calculation

$$[Vit]_{Stk} = \frac{W_s \times M \times S \times P \times 1{,}000}{Vol}$$

Where,

$[Vit]_{Stk}$:	Vitamin standard stock solution concentration, in µg/mL.
W_S:	Weight of standard in mg.
M:	Moisture content correction factor for the standard, if applicable.
S:	Stoichiometric correction factor, if applicable.
P:	Purity of standard as defined by the manufacturer.
1,000:	Units conversion factor, from mg to µg.
Vol:	Dissolution volume in mL.

2. Calculation of vitamin concentrations in the Mixed Working Standard (MWS)

$$[Vit]_{MWS} = [Vit]_{Stk} \times \frac{Vol}{10\,mL}$$

Where,

$[Vit]_{MWS}$:	Vitamin concentration in the Mixed Working Standard in ng/mL.
$[Vit]_{Stk}$:	Concentration of Vitamin Stock Standard in µg/mL
Vol:	Volume of stock solution added to MWS in µL.

3. Calculation of Working Standard concentration

$$[Vit]_{WSx} = \frac{Vol_{MWS} \times [Vit]_{MWS}}{500}$$

Where,

$[Vit]_{WSx}$: Vitamin concentration in the working standard in ng/mL.

$[Vit]_{MWS}$: Concentration of vitamin in the Mixed Working Standard in ng/mL.

Vol_{MWS}: Volume of the Mixed Working Standard fortified in working standard in µL.

500: This value is the dilution factor.

4. Vitamin concentration calculated in product from analytical result:

$$[Vit]_{sample} = \frac{[Vit]_{AS} \times RW \times 500}{SW \times PW}$$

Where,

$[Vit]_{sample}$: Vitamin concentration in product, µg/kg

$[Vit]_{AS}$: Vitamin mass in the analytical sample as calculated from calibration curve, ng/mL.

RW: Reconstitution weight (total), g. For direct weight (liquid) samples RW = 1.

SW: Analytical sample weight, g.

PW: Powder weight (for reconstituted samples), g. For liquid samples, this value is 1.

500: This value is the dilution factor.

5. For vitamins B_3 and B_6, the reported concentration of the individual forms is summed to report total. For example, concentration of nicotinamide and nicotinic acid are summed to report "Total B_3" and concentration of pyridoxal, pyridoxamine, and pyridoxine are summed to report "Total B_6." Thiamine and Riboflavin don't require this step.

References

AOAC SMPR 2015.002 (2015) *J. AOAC Int.* 98, 1094
AOAC SMPR 2015.003 (2015) *J. AOAC Int.* 98, 1096
AOAC SMPR 2015.004 (2015) *J. AOAC Int.* 98, 1098
AOAC SMPR 2015.005 (2015) *J. AOAC Int.* 98, 1100
J. AOAC Int. (future issue)

Posted: December 16, 2015

Table 2015.14. Conditions for MS transitions on a Waters TQ-S along with retention time windows (while mass transitions are expected to remain the same across instrument platforms, the other parameters may need to be adjusted to maximize sensitivity)

Compound	Function No.	Start (min)	End (min)	Molecular Ion	Fragment Ion	Cone Voltage	Collision Energy (V)	Dwell Time (sec)
Nicotinamide*	1	2.71	3.20	122.9	80.1	20.0	16.0	0.025
Nicotinamide	1	2.71	3.20	122.9	96.0	20.0	16.0	0.025
$^{2}H_4$-Nicotinamide*	1	2.71	3.20	127.0	84.0	20.0	16.0	0.025
$^{2}H_4$-Nicotinamide	1	2.71	3.20	127.0	100.0	20.0	16.0	0.025
Nicotinic Acid*	2	0.50	1.70	124.0	80.0	20.0	16.0	0.025
Nicotinic Acid	2	0.50	1.70	124.0	106.0	20.0	16.0	0.025
$^{2}H_4$-Nicotinic Acid*	2	0.50	1.70	128.0	84.1	20.0	16.0	0.025
$^{2}H_4$-Nicotinic Acid	2	0.50	1.70	128.0	109.0	20.0	16.0	0.025
Pyridoxal	3	1.76	2.70	168.0	94.0	20.0	22.0	0.025
Pyridoxal*	3	1.76	2.70	168.0	150.0	20.0	12.0	0.025
$^{2}H_3$-Pyridoxal	3	1.76	2.70	171.0	97.0	20.0	22.0	0.025
$^{2}H_3$-Pyridoxal*	3	1.76	2.70	171.0	153.0	20.0	12.0	0.025
Pyridoxamine	4	0.50	1.70	169.0	134.0	20.0	20.0	0.025
Pyridoxamine*	4	0.50	1.70	169.0	152.0	20.0	12.0	0.025
$^{2}H_3$-Pyridoxamine	4	0.50	1.70	172.0	136.0	20.0	20.0	0.025
$^{2}H_3$-Pyridoxamine*	4	0.50	1.70	172.0	155.0	20.0	12.0	0.025
Pyridoxine*	5	2.41	3.00	170.0	134.0	20.0	18.0	0.025
Pyridoxine	5	2.41	3.00	170.0	152.0	20.0	12.0	0.025
$^{13}C_4$-Pyridoxine*	5	2.41	3.00	174.0	138.0	20.0	18.0	0.025
$^{13}C_4$-Pyridoxine	5	2.41	3.00	174.0	156.0	20.0	12.0	0.025
Thiamine	6	3.01	3.60	265.1	81.0	20.0	30.0	0.025
Thiamine*	6	3.01	3.60	265.1	122.0	20.0	12.0	0.025
$^{13}C_4$-Thiamine	6	3.01	3.60	269.0	81.0	20.0	30.0	0.025
$^{13}C_4$-Thiamine*	6	3.01	3.60	269.0	122.0	20.0	12.0	0.025
Riboflavin	7	4.21	5.00	377.0	172.0	20.0	35.0	0.025
Riboflavin*	7	4.21	5.00	377.0	243.0	20.0	20.0	0.025
$^{13}C_4$,$^{15}N_2$-Riboflavin	7	4.21	5.00	383.0	175.0	20.0	35.0	0.025
$^{13}C_4$,$^{15}N_2$-Riboflavin*	7	4.21	5.00	383.0	249.0	20.0	20.0	0.025

*Indicates primary transition used in quantitation.

INFANT FORMULA AND ADULT NUTRITIONALS

Simultaneous Determination of Total Vitamins B_1, B_2, B_3, and B_6 in Infant Formula and Related Nutritionals by Enzymatic Digestion and LC-MS/MS: Single-Laboratory Validation, First Action 2015.14

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This method provides simultaneous determination of total vitamins B_1, B_2, B_3, and B_6 in infant formula and related nutritionals (adult and infant). The method was given First Action for vitamins B_1, B_2, and B_6, but not B_3, during the AOAC Annual Meeting in September 2015. The method uses acid phosphatase to dephosphorylate the phosphorylated vitamin forms. It then measures thiamine (vitamin B_1); riboflavin (vitamin B_2); nicotinamide and nicotinic acid (vitamin B_3); and pyridoxine, pyridoxal, and pyridoxamine (vitamin B_6) from digested sample extract by liquid chromatography-tandem mass spectrometry. A single-laboratory validation was performed on 14 matrixes provided by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) to demonstrate method effectiveness. The method met requirements of the AOAC SPIFAN *Standard Method Performance Requirement* for each of the three vitamins, including average over-spike recovery of 99.6 ± 3.5%, average repeatability of 1.5 ± 0.8% relative standard deviation, and average intermediate precision of 3.9 ± 1.3% relative standard deviation.

AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) released a call for methods for total vitamins B_1, B_2, B_3, and B_6 in infant formula and related nutritionals. In the European Union and other countries, label claim is regulated based upon total vitamin content and not just the fortified form. Historically, microbiological methods were used to estimate total vitamin. However, these methods are challenged with newer, more diverse nutritional products and are no longer considered the gold standard. Newer, chromatographic methods, especially with mass spectral detection, are quickly becoming the new standard because their specificity enables accurate quantitation across more complex and diverse matrixes. However, that specificity then requires explicit definition of the vitamin forms necessary for a "total" vitamin determination. SPIFAN gathered experts in industry, government, and academia to provide these definitions. Total vitamin B_1 is defined as the sum of thiamine, thiamine monophosphate, thiamine pyrophosphate, and thiamine triphosphate in the *Standard Method Performance Requirement* (SMPR®; 1). Total B_2 is defined as riboflavin, riboflavin-5′-phosphate, and flavin adenine dinucleotide (2). Total B_3 is defined as the sum of nicotinic acid and nicotinamide (3). Finally, total B_6 includes five forms: pyridoxine, pyridoxal, pyridoxal-5′-phosphate, pyridoxamine, and pyridoxamine-5′-phosphate (4).

SPIFAN defined forms of vitamins B_1, B_2, B_3, and B_6 that represent the major contributors to total vitamin concentration in formulas and enter the formulation through both fortification and from ingredient sources. For example, depending on the protein fraction of milk and the degree of processing, the contribution of inherent (unfortified) vitamin can be virtually 0 to >45% of the total value (5). But, regardless of source, the concentration of each vitamin must be verified to meet label claim. Remarkably, the necessary sample treatments and separation as described later are similar for the intended vitamins and thus lend themselves to simultaneous determination, saving both time and cost. Further, the availability of modern mass spectrometry (MS) instrumentation with electrospray ionization (ESI) facilitates simultaneous determination by removing remaining hurdles associated with detection. Suppression of ionization is problematic for quantitation with ESI, but is overcome with the use of stable-isotope labeled internal standards. The associated cost of isotopically labeled standards, although perceived as great, only adds a few cents to the cost of a sample because of the small amount necessary. By contrast, the syringe filter required to prevent clogging the liquid chromatography (LC) column adds about $1 (USD) to the cost of a sample.

The combined method was developed to measure thiamine, riboflavin, pyridoxamine, pyridoxal, pyridoxamine, nicotinic acid, and nicotinamide directly. Separation was achieved with 20 mM ammonium formate mobile phase without ion pairing agent. Thiamine is not well retained in reverse phase at low pH without an ion pairing agent (6). However, ion pairing agents bring additional challenges to LC-tandem MS (MS/MS) determination. Improved retention of thiamine has been previously demonstrated by increasing the mobile phase pH (6). In fact, there is a striking improvement in retention for many water-soluble vitamin under reverse-phase conditions at moderate pH (5–7). This improvement in retention was harnessed to achieve good method performance for a subset of the targeted vitamin forms; however, elution of phosphorylated compounds is notoriously difficult (8). The phosphate moiety complexes with Fe^{3+} and thus phosphate

Submitted for publication February 18, 2016.
The method was approved by the AOAC Expert Review Panel for SPIFAN Nutrient Methods as First Action.
The expert review panel invites method users to provide feedback on the First Action methods.
Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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DOI: 10.5740/jaoacint.15-0315

containing compounds tend to tail considerably with conventional chromatographic equipment because of stainless steel plumbing and column frits (8). Further, the phosphorylated forms of these vitamins tend to be a small fraction of the free-form thus putting pressure on accuracy of quantitation. Instead of trying to overcome the challenges of direct analysis of the phosphorylated forms, this method includes an overnight enzymatic digestion with acid phosphatase to hydrolyze the phosphate yielding the free vitamin forms (6). Papain and α-amylase are also included in the enzymatic cocktail to digest protein and complex carbohydrate, respectively, thus aiding reduction in sample extract complexity (9).

Method performance was demonstrated in SPIFAN II matrixes. SPIFAN II matrixes are a range of infant formula and adult nutritionals prepared by a number of the infant formula manufacturers to enable single- and multilaboratory validation of methods. SPIFAN II matrixes include partially hydrolyzed, milk-based infant formula powder; partially hydrolyzed, soy-based infant formula powder; milk-based toddler formula powder; two milk-based infant formula powders; a soy-based infant formula powder; child formula powder; elemental (extensively hydrolyzed) infant formula powder; infant formula powder with fructooligosaccharides and galactooligosaccharides; milk-based, ready-to-feed (RTF) infant formula; low fat, adult nutritional powder; high protein, RTF adult nutritional; and high fat, RTF adult nutritional. The matrixes also include the National Institute of Standards and Technology (NIST) Standard Reference Material (SRM) for infant formula, SRM 1849a; and five, unfortified, placebo matrixes matching a select subset of those previously mentioned: partially hydrolyzed, milk-based infant formula powder; partially hydrolyzed, soy-based infant formula powder; milk-based, RTF infant formula; high protein, RTF adult nutritional; and high fat, RTF adult nutritional. In these matrixes, precision averaged 3.9%, and over-spike recovery was generally 95–105%. Both free and phosphorylated forms were over-spiked as part of the recovery experiment. The method was demonstrated to meet all SPIFAN SMPR.

AOAC Official Method 2015.14
Simultaneous Determination of Total Vitamins B_1, B_2, and B_6 in Infant Formula and Related Nutritionals Enzymatic Digestion and LC-MS/MS

First Action 2015

[Applicable for simultaneous quantitation of total B_1, B_2, and B_6 in infant formula]

A. Principle

This method facilitates simultaneous quantitation of three water-soluble vitamins in infant formula and related nutritional products, including all SPIFAN SMPR relevant forms of vitamins B_1, B_2, and B_6. Samples are prepared by enzymatic digestion with papain and α-amylase to hydrolyze protein and complex carbohydrate and acid phosphatase to free phosphorylated vitamin forms. Stable-isotope labeled internal standards are incorporated into the sample preparation to correct for variability in both the sample preparation and instrument response. A series of six mixed working standard (MWS) solutions spanning two orders of magnitude in vitamin concentration are used to generate calibration curves based on the peak response ratio of the analyte to its stable-isotope labeled internal standard.

Prepared samples and working standard solutions are injected onto an ultra-high pressure liquid chromatograph (UPLC) interfaced to a triple-quadrupole mass spectrometer for analysis. The mass spectrometer is configured to monitor parent–daughter (precursor–fragment) ion pairs for each analyte and internal standard. This reaction forms the basis for method selectivity. Analytes are quantified by least-squares regression using the response ratio of the analyte to its internal standard.

B. Apparatus and Materials

(a) *Control sample.*—NIST SRM 1849a, or current lot. Store at 4°C. National Institute of Standards and Technology, Gaithersburg, MD.

(b) *Waters Acquity BEH C18 column.*—2.1 × 100, 1.7 µm. Part No. 186002352. Waters Corp., Milford, MA

(c) *UPLC system.*—Waters Acquity Classic, or equivalent. Waters

(d) *Tandem quadrupole mass spectrometer with ESI probe.*—Waters Xevo TQ-S, or equivalent. Waters

(e) *Analytical balances* (*3*).—(*1*) One capable of accurately weighing 5.00 mg (for standards), six-place balance.

(*2*) An analytical five-place balance for samples.

(*3*) A top loading two-place balance capable of weighing to several hundred grams.

(f) *Water purifier.*—Millipore Milli-Q Water Purification System, or equivalent. EMD Millipore, Billerica, MA.

(g) *Reciprocal shaking bath capable of maintaining 37°C.*

(h) *Bottle-top dispenser.*—Capable of dispensing volumes of approximately 24 mL.

(i) *pH meter.*—Capable of measuring a pH of 4.0–5.0.

(j) Karl Fischer for moisture determination in the thiamin hydrochloride United States Pharmacopeia (USP) standard. Metrohm AG, Riverview, FL.

(k) *Oven.*—For drying riboflavin USP standard at 105°C.

(l) *Vacuum chamber.*—For drying pyridoxine hydrochloride USP standard.

(m) *Vortex mixer.*

(n) *Multiposition magnetic stir plate.*

(o) *Teflon coated, magnetic stir bars.*

(p) *Hot plate with magnetic stirring.*

(q) *Room light shields.*—A.L.P. Protect-A-Lamp, UV cutoff at 460 nm, or equivalent. A.L.P Lighting Components, Inc. Chicago, IL.

(r) *Graduated cylinders.*—Various sizes, including 100, 500, 1000, and 2000 mL.

(s) *Beakers.*—Various sizes, including 100, 200, 400, 600, 1000, and 2000 mL.

(t) *Volumetric flasks.*—Various sizes, including 10, 25, 50, 100, 250, and 2000 mL.

(u) *Mobile phase bottles.*—Glass, various sizes, including 250, 500, 1000, and 2000 mL.

(v) *Disposable, plastic Pasteur pipets.*

(w) *Amber bottles.*—Volume capacity of 50 and 100 mL (for stock standard storage).

(x) *Weighing vessels.*—Various, including disposable weighing boats and glass weighing funnels.

(y) *Positive displacement pipets.*—Gilson Microman: 10, 100, 250, and 1000 µL; Part Nos. F148501, F148504, F148505, and F148506. Gilson, Inc. Middleton, WI.

(Z) *Positive displacement pipet tips.*—Gilson Capillary Piston: 10, 100, 250, and 1000 µL; Part Nos. F148312, F148314, F148014, and F148560. Gilson, Inc.

(aa) *Plastic syringes.*—3 mL.

(ab) *Syringe filters, PTFE 0.45 µm.*—Acrodisc 25 mm, or equivalent. Pall Corporation, Port Washington, NY.

(ac) *50 mL self-standing, plastic centrifuge tubes.*

(ad) *Autosampler vials.*—9 mm amber with screw top 12 × 32 mm presplit PTFE-silicon septa (Waters, Part No. 186000847C, or equivalent). Waters

(ae) *Teflon coated magnetic stir bars.*

C. Reagents

(a) *Nicotinamide.*—USP Reference Standard (U.S. Pharmacopeial Convention, Inc. Rockville, MD) Official Lot; Cat. No. 1462006. Store as indicated on label.

(b) *Niacin (nicotinic acid).*—USP Reference Standard Official Lot; Cat. No. 1461003. Stored as indicated on label.

(c) *Pyridoxine hydrochloride.*—USP Reference Standard Official Lot; Cat. No. 1587001. Store in desiccator protected from white light. Dry according to manufacturer's instructions before use.

(d) *Riboflavin.*—USP Reference Standard Official Lot; Cat. No. 1603006. Store in desiccator protected from white light. Dry according to manufacturer's instructions before use.

(e) *Thiamine hydrochloride.*—USP Reference Standard Official Lot; Cat. No. 1656002. Store in desiccator protected from white light. Measure the moisture content of the powder before use.

(f) *Pyridoxamine dihydrochloride.*—Fluka Analytical Standard, Cat. No. P9380. Sigma-Aldrich, St. Louis, MO.

(g) *Pyridoxal hydrochloride.*—Sigma, Cat. No. P9130. Sigma-Aldrich

(h) *$^{2}H_4$-nicotinamide.*—CDN Isotopes, Pointe-Claire, Quebec, Canada; Cat. No. D-3457.

(i) *$^{2}H_4$-nicotinic acid.*—CDN Isotopes; Cat. No. D-4368.

(j) *$^{13}C_4$-pyridoxine.*—Pyridoxine:HCl (4,5-bis(hydroxymethyl)-$^{13}C_4$); Cambridge Isotope Laboratory, Tewksbury, MA; Cat. No. CLM-7563.

(k) *$^{2}H_3$-pyridoxal.*—IsoSciences, King of Prussia, PA; Cat. No.7098.

(l) *$^{2}H_3$-pyridoxamine.*—IsoSciences; Cat. No. 7099.

(m) *$^{13}C_4$-thiamine chloride.*—IsoSciences, King of Prussia, PA; Cat. No. 9209.

(n) *$^{13}C_4$,$^{15}N_2$-riboflavin.*—IsoSciences, King of Prussia, PA., Cat. No. 7072.

(o) *Acid phosphatase, type II from potato, 0.5–3.0 U/mg.*—Sigma, Cat. No. P3752. Sigma-Aldrich

(p) *Papain from Carica papaya, ≥3 U/mg.*—Sigma, Cat. No. 76220. Sigma-Aldrich

(q) *α-Amylase from Aspergillus oryzae, 150 U/mg.*—Sigma, Cat. No. A9857. Sigma-Aldrich

(r) *Hydrochloric acid concentrated (12 M).*—American Chemical Society (ACS) grade, or equivalent.

(s) *Ammonium formate: for mass spectrometry (≥99.0%).*—Fluka 70221 or equivalent. Sigma-Aldrich

(t) *Glacial acetic acid.*—Sigma ACS reagent grade, or equivalent. Sigma-Aldrich, St. Louis, MO.

(u) *Formic acid.*—Sigma ACS reagent grade, or equivalent. Sigma-Aldrich, St. Louis, MO.

(v) *Laboratory water.*—18.0 MΩ, <10 ppb total organic carbon (TOC), or equivalent.

(w) *Methanol.*—Fisher Scientific (Franklin, MA) LC-MS/MS Optima grade or EMD (EMD Millipore) Omni-Solve LC-MS grade.

(x) *EDTA, disodium salt dihydrate.*—ACS grade (99–101%), or equivalent.

(y) *Potassium phosphate dibasic.*—ACS grade (>98%), or equivalent.

(z) *Metaphosphoric acid.*—ACS grade (33.5–36.5%), or equivalent.

(aa) pH 4.0, 7.0, and 10.0 buffer solutions for pH meter calibration.

(ab) *Phosphoric acid.*—85%, ACS grade, or equivalent.

(ac) *Potassium hydroxide.*—40%, ACS grade, or equivalent.

D. Standard and Solution Preparation

(a) *Mobile phase A.*—20 mM ammonium formate in water. Using a graduated cylinder, transfer 500 mL laboratory water to a mobile phase reservoir. Add 0.631 g of ammonium formate and mix well. Scale as needed. Expiration: 3 days.

(b) *Mobile phase B.*—Methanol.

(c) *Weak needle wash.*—10% methanol in water, expiration 3 months.

(d) *Strong needle wash.*—Methanol.

(e) *50 mM ammonium formate.*—Using a graduated cylinder, transfer 1400 mL laboratory water to an appropriate reservoir. Add 4.41 g of ammonium formate and mix well. 1400 mL is adequate for six working standards and 32 samples. Scale as needed. Expiration: 3 days.

(f) *Enzyme cocktail.*—Using a graduated cylinder, transfer 200 mL 50 mM ammonium formate buffer to an appropriate reservoir. Add 200 ± 10 mg acid phosphatase, 80 ± 5 mg α-amylase, and 400 ± 10 mg papain. Mix for 10 min with magnetic stir plate and stir bar. Adjust to pH 4.0–4.5 with formic acid (approximately 100 µL). 200 mL is adequate for six working standards and 32 samples. Scale as needed. Prepare fresh daily.

(g) *Stable-isotope labeled compounds.*—Individual, internal standard stock solutions.

(*1*) *$^{2}H_4$-nicotinamide stock solution (approximate concentration: 560 µg/mL).*—Weigh 14.0 ± 0.1 mg into a tared weighing vessel. Quantitatively transfer to a 25 mL volumetric flask with laboratory water and *quantum satis* (QS) with laboratory water. Mix well and transfer to a 50 mL amber bottle and store refrigerated (2–8°C). Expiration: Until exhausted or evidence of contamination.

(*2*) *$^{2}H_4$-nicotinic acid stock solution (approximate concentration: 500 µg/mL).*—Weigh 12.5 ± 0.1 mg into a tared weighing vessel. Quantitatively transfer to a 25 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 50 mL amber bottle and store refrigerated (2–8°C). Expiration: Until exhausted or evidence of contamination.

(*3*) *$^{13}C_4$-pyridoxine stock solution.*—Approximate concentration: 70 µg/mL. Weigh 7.0 ± 0.1 mg into a tared weighing vessel. Quantitatively transfer to a 100 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 100 mL amber bottle and store refrigerated (2–8°C). Expiration: Until exhausted or evidence of contamination.

(*4*) *$^{2}H_3$-pyridoxal stock solution.*—Approximate concentration: 40 µg/mL. Weigh 4.0 ± 0.1 mg into a tared weighing vessel. Quantitatively transfer to a 100 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 100 mL amber bottle and store refrigerated (2–8°C). Expiration: Until exhausted or evidence of contamination.

(*5*) *$^{2}H_3$-pyridoxamine stock solution.*—Approximate concentration: 40 µg/mL. Weigh 4.0 ± 0.1 mg into a tared weighing vessel. Quantitatively transfer to a 100 mL volumetric flask with laboratory water and QS with laboratory water. Mix well and transfer to a 100 mL amber bottle and store refrigerated (2°–8°C). Expiration: Until exhausted or evidence of contamination.

(*6*) *$^{13}C_4$-thiamine chloride stock solution.*—Approximate concentration: 100 µg/mL.

(*i*) *0.12 M HCl.*—Add approximately 300 mL water to a 500 mL graduated cylinder. Add 5.0 ± 0.1 mL conc. HCl and swirl to mix. Bring to 500 mL with laboratory water and mix well.

(*ii*) Weigh 5.0 ± 0.1 mg $^{13}C_4$-thiamine into a tared weighing vessel. Quantitatively transfer to a 50 mL volumetric flask with 0.12 N HCl and QS with 0.12 N HCl. Mix well and transfer to a 100 mL amber bottle and store refrigerated (2–8°C). Expiration: Until exhausted or evidence of contamination.

(*7*) *$^{13}C_4$,$^{15}N_2$-riboflavin stock solution.*—Approximate concentration: 73 µg/mL.

(*i*) *1.0% Acetic acid in water.*—Add approximately 30 mL water to a 500 mL graduate cylinder. Add 5.0 ± 0.1 mL glacial acetic acid and swirl to mix. Bring to 500 mL with laboratory water and mix well.

(*ii*) Weigh 7.3 ± 0.1 mg $^{13}C_4$,$^{15}N_2$-riboflavin into a tared weighing vessel. Quantitatively transfer to a 100 mL volumetric flask with 1.0% acetic acid and QS with 1.0% acetic acid. Mix well and transfer to a 100 mL amber bottle and store refrigerated (2–8°C). Expiration: Until exhausted or evidence of contamination.

(h) *Internal standard stock mixture (ISSM).*—Combine 2500 µL of 50 mM ammonium formate with 250 µL $^{2}H_4$-nicotinamide stock, 250 µL $^{2}H_4$-nicotinic acid stock, 250 µL $^{13}C_4$-pyridoxine stock, 200 µL $^{2}H_3$-pyridoxal stock, 50 µL $^{2}H_3$-pyridoxamine stock, 250 µL $^{13}C_4$-thiamine stock, and 250 µL $^{13}C_4$,$^{15}N_2$-riboflavin acid stock. Volume provides sufficient ISSM for six working standards and 32 samples. Scale as needed. Prepare fresh daily.

(i) *Phosphate buffer solution, pH 5.0.*—0.10 M potassium phosphate dibasic, 1% EDTA, 2% metaphosphoric acid.

(*1*) Weigh 20.0 ± 0.2 g EDTA into a tared weighing vessel and quantitatively transfer to a 2000 mL beaker containing approximately 1800 mL laboratory water and add a magnetic stir bar.

(*2*) Weigh 34.8 ± 0.1 g potassium phosphate dibasic into a tared weighing vessel and quantitatively transfer to the 2000 mL beaker already containing approximately 1800 mL laboratory water and EDTA. Mix by stirring on a magnetic stir plate until both the EDTA and potassium phosphate dibasic is completely dissolved.

(*3*) Weigh 40.0 ± 0.2 g metaphosphoric acid into a tared weighing vessel and quantitatively transfer to the 2000 mL beaker containing approximately 1800 mL laboratory water, EDTA, and potassium phosphate dibasic. Mix by stirring on a magnetic stir plate until the metaphosphoric acid is completely dissolved.

Adjust the pH of the solution to pH 5.00 ± 0.02 using 40% potassium hydroxide or 85% phosphoric acid. Quantitatively transfer the solution to a 2000 mL volumetric flask and dilute to volume with laboratory water. Expiration: 48 h.

(j) *Native compounds.*—Stock standard solutions.

(*1*) *Vitamin standard stock mixture (VSSM).*—Accurately weigh the indicated amounts for the following standards using separate weighing funnels or other appropriate weighing vessel and quantitatively transfer to a 100 mL volumetric flask using phosphate buffer (pH 5):

(*i*) *Niacinamide.*—70.5 ± 0.5 mg.

(*ii*) *Thiamine hydrochloride.*—10.5 ± 0.2 mg.

Determine the moisture of the USP thiamine hydrochloride reference standard as directed on the container immediately before weighing. The percent moisture determined for the reference standard is used to calculate the concentration of thiamine in the VSSM.

(*iii*) *Riboflavin.*—7.0 ± 0.2 mg. Dry an appropriate amount of the USP riboflavin reference standard at 105 (±1)°C for 2 h (±10 min) before weighing.

(*iv*) *Pyridoxine hydrochloride.*—10.8 ± 0.2 mg. Dry an appropriate amount of the USP pyridoxine hydrochloride reference standard over indicating absorbent in vacuo for 4 h before weighing.

QS to volume with phosphate buffer (pH 5) solution. Heat and slowly stir until the standards have completely dissolved (riboflavin dissolves more slowly) and the solution is clear. Do not heat the solution for more than 40 min and do not exceed 90°C. Store refrigerated (2–8°C). Expiration: 3 months.

(*2*) *Nicotinic acid stock solution (550 µg/mL).*—Accurately weigh 13.7 ± 0.1 mg USP niacin (nicotinic acid, Cat. No. 1461003) reference standard. Quantitatively transfer the nicotinic acid to a 25 mL volumetric flask. Add laboratory water to a total volume of about 20 mL and swirl until completely dissolved. Bring to volume with laboratory water. Mix well. Expiration: 3 months.

(*3*) *Pyridoxal stock solution.*—140 µg/mL. Accurately weigh 17.0 ± 0.5 mg pyridoxal dihydrochloride standard. Quantitatively transfer to a 100 mL volumetric flask. Add laboratory water to a total volume of about 70 mL and swirl until completely dissolved. Bring to volume with laboratory water. Mix well. Expiration: 3 months.

(*4*) *Pyridoxamine stock solution.*—160 µg/mL. Accurately weigh 23.0 ± 0.5 mg pyridoxamine hydrochloride standard. Quantitatively transfer to a 100 mL volumetric flask. Add laboratory water to a total volume of about 70 mL and swirl until completely dissolved. Bring to volume with laboratory water. Mix well. Expiration: 3 months.

(k) *MWS.*—Combine 500 µL VSSM, 25 µL pyridoxamine stock, 25 µL pyridoxal stock, and 65 µL nicotinic acid stock solutions in a 10 mL volumetric flask containing approximately 5 mL of 50 mM ammonium formate. Bring to volume with 50 mM ammonium formate and mix well. Prepare fresh daily.

(l) *Working standard solution preparation.*—(*1*) *WS1.*—Add 20 µL MWS and 980 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

(*2*) *WS2.*—Add 50 µL MWS and 950 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

(*3*) *WS3.*—Add 100 µL MWS and 900 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

(*4*) *WS4.*—Add 200 µL MWS and 800 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

(*5*) *WS5.*—Add 500 µL MWS and 500 µL of 50 mM ammonium formate to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

(*6*) *WS6.*—Add 1000 µL MWS to a 50 mL centrifuge tube. Add 100 µL of ISSM, and vortex to mix. Prepare fresh daily.

E. Procedure

(a) *Sample preparation*.

(*1*) *For powdered products.*—Using a tared beaker or low-density polyethylene cup, weigh 250.0 ± 0.3 g of sample. Record the weight to at least four significant figures. This is the powder weight. Add room temperature laboratory water to bring the total reconstituted sample weight (to include the product weight) to 225 ± 2 g. Record the weight to at least four significant figures. This is the reconstitution weight. Carefully add a stir bar so as not to splash the liquid from the beaker/cup and place it onto a stir plate. Set the stir plate to stir the sample as fast as possible without causing the sample to splatter or froth. Powder samples should stir for at least 10 min but not more than 30 min.

(*2*) *For reconstituted powders and liquid products.*—Using a tared, 50 mL centrifuge tube, weigh the appropriate sample amount (1.000 ± 0.100 g for infant formula, 0.500 ± 0.050 g for pediatric formulas and the NIST SRM, and 0.250 ± 0.050 g for adult nutritionals). Record the weight to 0.0001 g. This is the sample weight. Add 100 µL of the ISSM via positive-displacement pipet. Vortex to mix.

(b) *Enzymatic digestion.*—Add 5 mL of enzyme cocktail to all prepared samples and working standards. Cap and vortex immediately. Incubate at 37°C overnight with agitation in water bath shaker. Remove from water bath, and add 50 mM ammonium formate buffer to bring volume to approximately 30 mL and vortex to mix. Filter approximately 2 mL aliquot of the sample extract into an appropriate size vial using a 0.45 µm PTFE syringe filter. Transfer 60 µL of filtrate to an autosampler vial with 940 µL of 50 mM ammonium formate buffer. Cap and vortex. The sample is ready for analysis. Samples have been determined to be stable for at least 48 h at room temperature.

(c) *LC-MS/MS analysis*.

(*1*) *UPLC conditions.*—Place freshly prepared mobile phases, weak needle wash, and strong needle wash onto the LC system. Purge old solvents from the solvent lines and needle washes. Injection volume is 10 µL and column temperature is 40°C. Mobile phase flow rate is 0.350 mL/min. Hold at 99% mobile phase A and 1.0% mobile phase B for 0.50 min, then ramp to 8.0% B over 2.00 min, ramp to 90% B over the next 2.50 min, and hold at 90% B for 1.00 min. Return to 99% mobile phase A and 1.0% mobile phase B over 0.10 min and hold for 1.9 min for re-equilibration. Total gradient program is 8.00 min long. An example chromatogram is given in Figure 1 for reference.

(*2*) *MS tune conditions.*—Clean the sample cone and MS source with 5% formic acid before analysis. Tune conditions can vary between instrument models and appropriate balance must be struck to achieve adequate signal for each compound. Appropriate conditions must be determined experimentally for each instrument model. On a Waters TQ-S, ionization is performed by ESI+ at 2.5 kV. Additional tune conditions include: source offset of 50 V, ion block temperature of 150°C, desolvation gas temperature of 500°C, desolvation gas flow of 800 L/h, cone gas flow of 150 L/h, nebulizer gas pressure of 7.00 bar, collision gas flow of 0.15 mL/min with argon. Both quadrupoles are set to unit mass resolution.

(*3*) *Mass transitions.*—Mass transitions for each vitamin and its corresponding internal standard are given in Table 1. Retention time windows are also given in the table. Like the tune parameters, these parameters may need adjusted based upon instrument model.

(*4*) *UPLC-MS/MS equilibration.*—The instrument should be held at initial conditions (with mobile phase flow on and MS at temperature) for 30–60 min before injection. Alternatively, 6–10 blank injections at the start of a sequence can be used for the same purpose.

(d) *Quality control*.

(*1*) Blanks of 50 mM ammonium formate need to bracket each calibration curve to enable check for laboratory background and instrumental carryover. Background should be no more than 5% of the signal for the lowest working standard.

(*2*) *Calibration curve.*—Calibration curves are set up to bracket the sample injections. Calibration residuals (relative error from known concentration) are expected to be ≤20% for pyridoxal and ≤8% for the other vitamins. A standard injection outside of this range can be excluded with evidence of a standard preparation error in a single calibration level leading to a high or low response for all vitamins or evidence of a one-off instrumental error, such as a missed injection.

(*3*) *Laboratory control.*—NIST SRM 1849a, or current lot, serves as a control sample and should be prepared and analyzed with each sample set. The control result for each analyte must be within limits established by a control chart. By and large, the levels reported by this method are within the NIST certified range because of the minute concentration of phosphorylated vitamin forms in SRM 1849a.

F. Calculations

(a) Vitamin stock solutions concentration calculation:

$$[Vit]_{Stk} = \frac{W_s \times M \times S \times P \times 1{,}000}{Vol}$$

where $[Vit]_{Stk}$ = vitamin standard stock solution concentration, in µg/mL; W_S = weight of standard in mg; M = moisture content correction factor for the standard, if applicable; S = stoichiometric correction factor, if applicable; P = purity of standard as defined by the manufacturer; 1,000 = units conversion factor, from mg to µg; and Vol = dissolution volume in mL.

(b) Calculation of vitamin concentrations in the MWS:

$$[Vit]_{MWS} = [Vit]_{Stk} \times \frac{Vol}{10\ mL}$$

where $[Vit]_{MWS}$ = vitamin concentration in the MWS in ng/mL; $[Vit]_{Stk}$ = concentration of vitamin stock standard in µg/mL; and Vol = volume of stock solution added to MWS in µL.

(c) Calculation of working standard concentration:

$$[Vit]_{WSx} = \frac{Vol_{MWS} \times [Vit]_{MWS}}{500}$$

Table 1. Conditions for MS transitions on a Waters TQ-S are given along with retention time windows

Compound	Function No.	Start, min	End, min	Molecular ion	Fragment ion	Cone voltage	Collision energy (V)	Dwell time, s
Nicotinamide[a]	1	2.71	3.20	122.9	80.1	20.0	16.0	0.025
Nicotinamide	1	2.71	3.20	122.9	96.0	20.0	16.0	0.025
$^{2}H_4$-nicotinamide[a]	1	2.71	3.20	127.0	84.0	20.0	16.0	0.025
$^{2}H_4$-nicotinamide	1	2.71	3.20	127.0	100.0	20.0	16.0	0.025
Nicotinic acid[a]	2	0.50	1.70	124.0	80.0	20.0	16.0	0.025
Nicotinic acid	2	0.50	1.70	124.0	106.0	20.0	16.0	0.025
$^{2}H_4$-nicotinic acid[a]	2	0.50	1.70	128.0	84.1	20.0	16.0	0.025
$^{2}H_4$-nicotinic acid	2	0.50	1.70	128.0	109.0	20.0	16.0	0.025
Pyridoxal	3	1.76	2.70	168.0	94.0	20.0	22.0	0.025
Pyridoxal[a]	3	1.76	2.70	168.0	150.0	20.0	12.0	0.025
$^{2}H_3$-pyridoxal	3	1.76	2.70	171.0	97.0	20.0	22.0	0.025
$^{2}H_3$-pyridoxal[a]	3	1.76	2.70	171.0	153.0	20.0	12.0	0.025
Pyridoxamine	4	0.50	1.70	169.0	134.0	20.0	20.0	0.025
Pyridoxamine[a]	4	0.50	1.70	169.0	152.0	20.0	12.0	0.025
$^{2}H_3$-pyridoxamine	4	0.50	1.70	172.0	136.0	20.0	20.0	0.025
$^{2}H_3$-pyridoxamine[a]	4	0.50	1.70	172.0	155.0	20.0	12.0	0.025
Pyridoxine[a]	5	2.41	3.00	170.0	134.0	20.0	18.0	0.025
Pyridoxine	5	2.41	3.00	170.0	152.0	20.0	12.0	0.025
$^{13}C_4$-pyridoxine[a]	5	2.41	3.00	174.0	138.0	20.0	18.0	0.025
$^{13}C_4$-pyridoxine	5	2.41	3.00	174.0	156.0	20.0	12.0	0.025
Thiamine	6	3.01	3.60	265.1	81.0	20.0	30.0	0.025
Thiamine[a]	6	3.01	3.60	265.1	122.0	20.0	12.0	0.025
$^{13}C_4$-thiamine	6	3.01	3.60	269.0	81.0	20.0	30.0	0.025
$^{13}C_4$-thiamine[a]	6	3.01	3.60	269.0	122.0	20.0	12.0	0.025
Riboflavin	7	4.21	5.00	377.0	172.0	20.0	35.0	0.025
Riboflavin[a]	7	4.21	5.00	377.0	243.0	20.0	20.0	0.025
$^{13}C_4$,$^{15}N_2$-riboflavin	7	4.21	5.00	383.0	175.0	20.0	35.0	0.025
$^{13}C_4$,$^{15}N_2$-riboflavin[a]	7	4.21	5.00	383.0	249.0	20.0	20.0	0.025

Although the mass transitions are expected to remain the same across instrument platforms, the other parameters may need to be adjusted to maximize sensitivity.

[a] Indicates primary transition used in quantitation.

where $[Vit]_{WSx}$ = vitamin concentration in the working standard in ng/mL; $[Vit]_{MWS}$ = concentration of vitamin in the MWS in ng/mL; Vol_{MWS} = volume of the MWS fortified in working standard in µL; and 500 = dilution factor.

(d) Vitamin concentration calculated in product from analytical result:

$$[Vit]_{sample} = \frac{[Vit]_{AS} \times RW \times 500}{SW \times PW}$$

where $[Vit]_{sample}$ = vitamin concentration in product, µg/kg; $[Vit]_{AS}$ = vitamin mass in the analytical sample as calculated from calibration curve, ng/mL; RW = reconstitution weight (total), g, for direct weight (liquid) samples RW = 1; SW = analytical sample weight, g; PW = powder weight (for reconstituted samples), g, for liquid samples, this value is 1; and 500 = dilution factor.

(e) For vitamins B_3 and B_6, the reported concentration of the individual forms is summed to report total. For example, concentration of nicotinamide and nicotinic acid are summed to report "Total B_3" and concentration of pyridoxal, pyridoxamine, and pyridoxine are summed to report "Total B_6." Thiamine and riboflavin do not require this step.

Validation

Method performance was demonstrated against predefined suitability criteria for these vitamins published in SMPRs (1–4). Although each SMPR is slightly different, methods for B_1, B_2, B_3, and B_6 are required to achieve repeatability of ≤5% RSD, reproducibility of ≤10% RSD, and over-spike recovery of 90–110%. This method met each of these requirements except reproducibility, which was not evaluated. Instead, intermediate precision is given and suggests the reproducibility requirement will be met upon multilaboratory evaluation. Additional measures of method performance are also discussed, including: linearity, specificity, and robustness.

Linearity

This method includes six working standards to bracket the distribution of vitamin concentrations in SPIFAN II products. Calibration curves were generated at the beginning and end of each analysis as required by the method. Each standard in the curve has its percent deviation calculated as the percent difference between the calculated concentration and the true concentration. Percent deviation of ±4% is typical for vitamins B_1, B_2, B_3, pyridoxamine, and pyridoxine; and percent deviation of ±11% is typical for pyridoxal, which has lower response. Good performance was observed (Table 2).

Accuracy

Accuracy was evaluated by over-spike recovery in the five SPIFAN II placebos and three of select SPIFAN II products (Table 3). The placebos were manufactured without fortification of vitamins and minerals, but do contain some inherent vitamins and minerals by contribution of the proteins, carbohydrates, and fats. An additional three fortified SPIFAN II samples were chosen for over-spike studies because they were different enough from the placebos to warrant additional inquiry: partially hydrolyzed, milk-based infant formula powder; partially hydrolyzed, soy-based infant formula powder; and soy-based infant formula powder. For over-spike recovery, each matrix was spiked at both low and high levels corresponding to approximately 50% and 200% of fortification, respectively. Each spike level was performed with independent sample preparation, and the experiment was repeated on three different days for a total of $n = 6$ data points at each level in each matrix. Recovery was calculated as the reported concentration divided by the inherent contribution plus the amount spiked. All vitamin forms required by the SMPRs were combined in the spiking solution except thiamine triphosphate, which was not available for purchase. Over-spike levels for each form were targeted to mimic ratios previously reported in infant formulas and milk: thiamine monophosphate and thiamine diphosphate were spiked at 12.3% and 8.6% of total B_2; riboflavin phosphate and flavin adenine dinucleotide were spiked at 18.1% and 8.8% of total B_2; nicotinic acid was spiked at 7.2% of total B_3; and pyridoxal and pyridoxal-5′-phosphate were spiked at 4.9% and 4.3% and pyridoxamine and pyridoxamine-5′-phosphate were spiked at 5.8% and 5.0% of total spiked B_6. On an RTF concentration basis, over-spikes were 2.60 and 21.0 µg/100 g of total pyridoxal; 3.00 and 24.0 µg/100 g of total pyridoxamine; 22.5 and 180 µg/100 g of total pyridoxine; 31.5 and 250 µg/100 g of total thiamine; 24.0 and 190 µg/100 g of total riboflavin; and 190 and 1500 µg/100 g of total B_3. Good over-spike recovery was demonstrated (Table 3).

Precision

Repeatability and intermediate precision were determined from six independent preparations of all 14 products over 6 days. The experiments were performed by two analysts and on one instrument. Repeatability and intermediate precision are reported as %RSD in Tables 4 and 5. SPIFAN SMPRs for repeatability and reproducibility are ≤5% and ≤10% RSD, respectively.

Robustness

Method robustness was evaluated during development by using three analysts and two instruments. The method was tested over 6 days as well with independent preparations for each data point, and accuracy was done over an additional three days for each matrix. Data were collected over the course of about 8 weeks. Given these variables, precision and accuracy were excellent suggesting good method robustness. Further, a review of sample weights collected during sample preparation show that the powder weight varied by up to 6%, the reconstitution weight varied by up to 8%, and the liquid sample weight varied by up to 9%. Given the demonstrated precision and accuracy, this method shows good robustness toward sample size variation.

Within a run, there is notable signal suppression in some matrixes. Suppression is most easily observed by noting the absolute change in the internal standard intensity in samples compared with standards. The degree of suppression is matrix- and vitamin-dependent and ranged from negligible up to loss of 50% of the signal. Ion suppression is not uncommon with ESI, and necessitates the use of stable-isotope labeled internal

Table 2. Calibration curve % deviation from true concentration is reported at each calibration level[a]

Standard	Overall (n = 12)	Thiamine	Riboflavin	Niacin	Nicotinic acid	Pyridoxal	Pyridoxamine	Pyridoxine
WS1	Recovery (%)	99.2	99.6	98.3	100.5	104.4	101.3	100.9
	RSD (%)	3.5	6.6	5.9	7.3	15.4	7.2	2.3
WS2	Recovery (%)	100.4	99.9	100.8	99.1	97.9	100.3	98.8
	RSD (%)	3.2	4.9	5.1	4.7	10.2	3.5	1.6
WS3	Recovery (%)	100.3	100.3	101.2	100.0	95.3	97.5	100.0
	RSD (%)	2.1	3.7	3.2	2.2	10.7	4.6	1.3
WS4	Recovery (%)	100.5	100.6	100.5	100.4	104.3	99.8	100.4
	RSD (%)	2.2	3.4	3.4	2.6	9.9	3.6	2.0
WS5	Recovery (%)	99.6	99.6	99.3	99.8	98.9	100.7	99.9
	RSD (%)	2.4	1.9	2.5	1.8	10.9	4.0	1.4
WS6	Recovery (%)	100.1	100.1	100.2	100.0	100.1	99.8	100.0
	RSD (%)	2.2	1.1	3.0	1.8	8.7	3.2	1.1

[a] The reported value is averaged across 6 days and reported along with %RSD.

Table 3. Accuracy is expressed in terms of the average over-spike recovery in select matrixes

	Total B_1		Total B_2		Total B_3		Total B_6	
Matrix	%Rec[a]	%RSD	%Rec	%RSD	%Rec	%RSD	%Rec	%RSD
Adult nutritional RTF, high-fat; placebo								
Low QC	94.9	4.9	97.2	1.6	102.1	7.8	100.2	3.2
High QC	93.4	5.7	95.5	1.7	103.1	6.8	99.5	2.7
Adult nutritional RTF, high-protein; placebo								
Low QC	97.7	4.3	96.5	2.2	101.6	5.9	96.8	2.4
High QC	95.4	5.5	98.4	2.7	102.9	3.8	98.8	2.7
Child formula powder, placebo								
Low QC	99.0	5.7	98.0	2.7	105.4	6.3	99.7	1.8
High QC	97.0	7.0	98.9	2.8	105.7	5.3	99.1	1.9
Infant elemental powder, placebo								
Low QC	93.7	6.4	95.1	1.8	104.5	6.8	97.9	2.1
High QC	92.4	7.8	96.7	1.0	103.8	4.9	98.4	3.4
Infant formula powder partially hydrolyzed, milk-based								
Low QC	100.5	1.9	100.2	1.6	103.8	1.2	99.1	1.5
High QC	101.3	1.8	100.0	2.4	111.7	1.7	100.1	2.9
Infant formula powder partially hydrolyzed, soy-based								
Low QC	100.7	1.5	100.7	2.1	102.0	0.9	99.3	0.9
High QC	103.1	0.6	99.6	2.0	106.7	1.1	99.8	2.4
Infant formula powder, soy-based								
Low QC	99.3	1.4	95.7	4.4	101.1	1.7	98.9	1.3
High QC	96.6	3.4	96.8	2.9	100.1	2.9	97.2	2.0
Infant formula RTF, milk-based; placebo								
Low QC	96.9	4.3	100.7	1.8	105.3	5.7	98.6	2.8
High QC	95.5	6.1	97.9	3.2	103.3	6.9	99.6	3.8

A cocktail including all vitamin forms listed in the SMPRs was spiked at approximately 50 and 200% of the fortification level.

[a] %Rec = Percent recovery.

standards for good method precision and accuracy. Standard addition is an alternative means to quantitate without stable-isotope labeled internal standards, but was not evaluated in this method because it is not practical when many different matrix types are tested within a single run.

Despite the degree of signal suppression, ion ratio stability between the two transitions was demonstrated to be good across matrixes. For vitamin forms with modest signal intensity (pyridoxine, thiamine, nicotinamide, and riboflavin), ion ratios in the samples averaged 101 ± 3% of the ion ratio in the standards.

Table 4. Repeatability for six independent preparations is expressed as %RSD

Matrix	Total B_1	Total B_2	Total B_3	Total B_6
S01: 1849a	1.0	1.8	1.2	1.0
S02: Infant formula powder partially hydrolyzed, milk-based	0.7	1.7	1.6	1.5
S03: Infant formula powder partially hydrolyzed, soy-based	0.8	2.3	1.2	1.3
S04: Toddler formula powder, milk-based	1.2	2.8	1.0	4.2
S05: Infant formula powder, milk-based	1.1	2.3	1.3	1.1
S06: Adult nutritional powder, low-fat	1.8	3.8	1.7	1.5
S07: Child formula powder	1.6	2.6	1.3	0.8
S08: Infant elemental powder	0.6	2.6	0.6	1.6
S09: Infant formula powder FOS/GOS-based[a]	1.1	2.6	1.0	0.8
S10: Infant formula powder, milk-based	1.0	2.3	1.0	1.1
S11: Infant formula powder, soy-based	0.9	1.4	1.0	1.2
S12: Infant formula RTF, milk-based	0.9	1.6	0.9	1.0
S13: Adult nutritional RTF, high-protein	1.8	2.4	1.9	2.9
S14: Adult nutritional RTF, high-fat	1.0	1.6	1.0	1.5

[a] FOS/GOS = Fructo-oligosaccharides/galacto-oligosaccharides.

Table 5. Intermediate precision for six independent preparations is expressed as %RSD

Matrix	Total B_1	Total B_2	Total B_3	Total B_6
S01: 1849a	3.0	4.7	4.5	4.0
S02: Infant formula powder partially hydrolyzed, milk-based	3.7	3.9	4.6	3.6
S03: Infant formula powder partially hydrolyzed, soy-based	2.5	4.5	2.3	3.3
S04: Toddler formula powder, milk-based	3.5	5.6	2.4	6.2
S05: Infant formula powder, milk-based	2.8	5.4	2.0	3.0
S06: Adult nutritional powder, low-fat	3.4	9.0	2.8	5.1
S07: Child formula powder	3.6	5.3	2.4	3.6
S08: Infant elemental powder	3.0	5.7	1.4	4.3
S09: Infant formula powder, FOS/GOS-based[a]	4.0	4.1	2.3	4.1
S10: Infant formula powder, milk-based	4.1	4.5	1.6	4.5
S11: Infant formula powder, soy-based	3.1	3.7	2.2	3.5
S12: Infant formula RTF, milk-based	4.0	5.8	3.3	5.7
S13: Adult nutritional RTF, high-protein	4.1	5.6	3.6	6.0
S14: Adult nutritional RTF, high-fat	3.2	4.2	3.2	5.0

[a] FOS/GOS = Fructo-oligosaccharides/galacto-oligosaccharides.

Thiamine was a notable exception because of chromatographic interference in the first transition for the $^{13}C_4$-thiamine internal standard (Table 1). This chromatographic interference does not impact method accuracy because the first transition is not used for quantitation. However, it does mean that ion ratio suitability criteria cannot be specified for the thiamine internal standard. Ion ratios for the lower intensity vitamins (pyridoxal, pyridoxamine, and nicotinic acid) had a larger degree of variation because of the lower signal intensity. They averaged 102 ± 12% of the ion ratio in the standards. During the over-spike studies in which the signal intensity was higher, the variation in the ion ratio was reduced and approached the ±3% level of the more abundant vitamin forms.

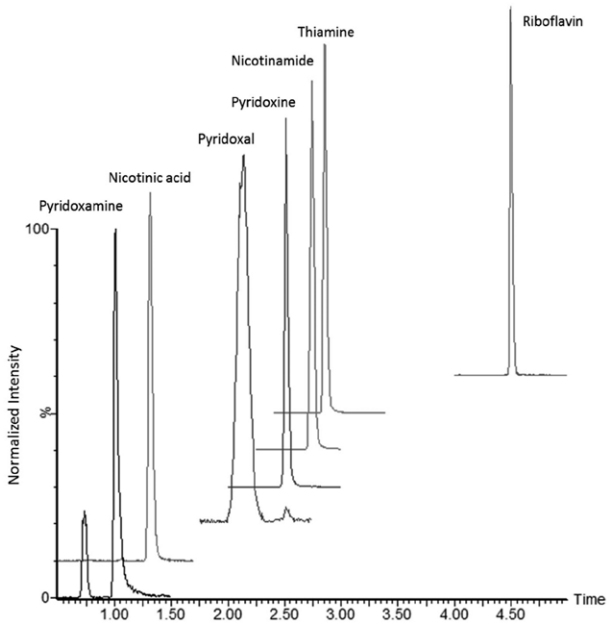

Figure 1. A chromatogram for the seven vitamin forms in the child formula powder. The data are unsmoothed, and the intensity of each peak is normalized to aid visualization. There is more than two orders of magnitude difference in signal intensity, which makes the small features such as pyridoxamine and pyridoxal difficult to see when all are plotted on the same scale.

Finally, the choice of enzyme is important for method performance. During method development, two different acid phosphatases were investigated, one from Roche Diagnostics and one from Sigma-Aldrich. The acid phosphatase from Roche did not fully hydrolyze pyridoxamine-5′-phosphate and generally recovered about 50% of the over-spiked level. Further, it generated significant amounts of nicotinic acid during digestion on the order of up to 10% of the total vitamin B_3. Although the source of the nicotinic acid is not entirely clear, it appears to result from conversion of nicotinamide to nicotinic acid because the total B_3 concentration (sum of nicotinamide and nicotinic acid) did not increase significantly in the three matrixes studied in detail. The method was validated using the acid phosphatase from Sigma-Aldrich. This acid phosphatase contains higher levels of pyridoxamine and pyridoxal, riboflavin, and nicotinic acid; but was chosen because it eliminates problems with nicotinic acid conversion and pyridoxamine-5′-phosphate recovery. The background vitamin levels in the Sigma-Aldrich acid phosphatase as a percent of their concentrations in SRM 1849a are 0.1% thiamine, 2.8% riboflavin, 0.2% nicotinamide, 18% nicotinic acid, 6.2% pyridoxal, 0.5% pyridoxamine, and 0.2% pyridoxine. However, these data need additional context. Nicotinic acid, pyridoxal, and pyridoxamine in SRM 1849a are virtually absent. From a total vitamin perspective, the overall contribution of vitamins from the enzyme in SRM 1849a is 0.1% total B_1, 2.8% total B_2, 0.3% total B_3, and 0.5% total B_6. Despite the small contribution from the enzyme, the standards are prepared as samples to mitigate any impact on method accuracy. The development work presented serves as caution: substitution of enzymes for other than those specified by this method may be deleterious to method performance. The use of an alternative enzyme would require significant investigation to the efficacy, digestion, and background contribution of vitamins to ensure adequate method performance.

Conclusions

This enzymatic digestion LC-MS/MS method provides simultaneous quantitation of vitamins B_1, B_2, B_3, and B_6; and was given First Action status for vitamins B_1, B_2, and B_6. Method performance was demonstrated over 6 days in 14 different matrixes with three analysts and on two instruments. Intermediate precision averaged 3.9% and over-spike recovery was generally 95–105% for all four vitamins.

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AOAC Official Method 2015.15
Nitrogen, Phosphorus and Potassium Release Patterns
of Slow- and Controlled-Release Fertilizers
First Action 2015

A. Scope

This method is applicable for the determination of N, P and K in slow-release and controlled-release fertilizers.

B. Principle

In Alternative A, a representative unground test portion is exposed to ambient temperature extractions of a solvent in a biologically active sandy soil medium. In Alternative B, a representative unground test portion is exposed to increasingly aggressive solvent extractions. Extractions are designed to extract and isolate nutrients becoming available over time. Each extract is analyzed by AOAC procedures for the nutrient of interest (total N, P and K). Along with analysis of total nutrients and reference materials, data are used to develop information specific to the cumulative percentage of nutrient released over time.

Alternative A: 180-Day Extraction at Ambient Temperature

C. Apparatus

(a) Extraction columns (incubation lysimeters; *see* Figure **2015.15A**) are constructed of PVC pipe (30 × 7.5 cm) fitted with a fiberglass mat in the bottom held in place by a 7.5 id PVC cap. The cap is fitted with a barbed plastic fitting, and vacuum tubing attached for leachate collection. A PVC cap is used on the top with no hole, but with a coating of stopcock grease to cap the lysimeter; all columns are supported on wood frame.

(b) A 50 mL beaker placed in the headspace of each incubation lysimeter.

(c) Filtering flasks with a one-hole stopper is placed beneath the leaching columns and attached to the vacuum tubing. A pinch clamp is used to prevent leaks when filtration and leachate collection is complete.

(d) Vacuum manifold and tubing connecting each flask to a standard laboratory vacuum pump.

(e) Riffle, gated or rotary.

D. Reagents and Reference Materials

(a) *Extraction solution*.—0.01% (w/v) citric acid (2 g/20 L deionized water).

(b) *Ammonia trap solution*.—0.2 M H_2SO_4 solution.

(c) *Loamy, siliceous, hyperthermic, Grossarenic Paleudult soil*.—Arredondo fine sand. Particle size analysis is shown in Table **2015.15A**.

(d) *Uncoated quartz sand*.—Topdress sand (noncoated quartz), 20/30 silica sand. Available from Edgar Minerals Inc., Edgar, FL and Standard Sand and Silica Co., Lynne, FL. Particle size analysis is shown in Table **2015.15B**.

(e) *Soil media*.—Mixture of 1710 g uncoated quartz sand (United States Gold Association Greens Mix), [D(**d**)] and 90 g loamy siliceous, hyperthermic, Grossarenic Paleudult soil [D(**c**)] or similar type of local soil acting as a microbial inoculum.

E. Sample Preparation of Granular Materials

Using a gated riffle splitter, reduce laboratory sample to yield an unground representative test portion containing approximately 450 mg of total N to mix thoroughly with the soil/sand mixture.

F. Procedure

Test portions from each material to be tested are placed in incubation columns held at room temperature (20–25°C). The sand/soil/test portion mixture is brought to 10% gravimetric moisture by adding 180 mL 0.01% citric acid. A 50 mL beaker containing 20 mL 0.2 M H_2SO_4 is placed in the headspace of the column as an ammonia trap. The solution in the ammonia trap is replaced and analyzed for NH_4-N by titration every 7 days. After 7, 14, 28, 56, 84, 112, 140, and 180 days, each column is leached at the same time of day with one pore volume (500 mL) 0.01% citric acid using a vacuum manifold. Vacuum is pulled for 2 min to ensure all free extraction solution is removed. Mix well and transfer to a 250 mL graduated cylinder. Record the leachate volume and remove aliquots to test for total N. In addition, measure the pH and EC of the leachate. Retain the remaining leachate in reserve in case an additional or recheck analysis is required. Store in dark bottles and freeze if retained for more than 7 days. (*Note*: If no volatile N is detected in the ammonia trap during the first two sampling periods, the NH_4 trap can be removed and analysis discontinued.)

G. Analytical Determinations

(a) Determine total N on each of the extracts obtained using AOAC Method **993.13** (combustion) or **978.02** (modified comprehensive; 1), or equivalent, applicable methods validated in your laboratory. Utilize an applicable method matched reference material in each run. Utilize at least three standards appropriate for the range of extract concentrations. Typically a combination of 10, 100, and 1000 mg N/L cover the range of N in the extracts.

(b) Determine total phosphate (as P_2O_5) using AOAC Method **962.02** or AOAC Method **978.01** (1) or equivalent, applicable methods validated in your laboratory. Utilize applicable method-matched reference material in each run. Utilize internal reference standard appropriate for the range of the sample extracts; typically 100, 1000, and 10 000 mg P_2O_5/L will cover the full range of P_2O_5 concentrations.

(c) Determine soluble potash (as K_2O) using AOAC Method **958.02** (STPB) or AOAC Method **983.02** (flame photometry; 1) or equivalent, applicable methods validated in your laboratory. Utilize applicable method-matched reference material in each run of samples. Utilize internal reference standard appropriate for the range of the sample extracts; typically 100, 1000, and 10 000 mg. K_2O/L will cover the full range of K_2O concentrations.

Nomenclature for Extraction Calculation Equations

Time is measured in days and is expressed in the extract identifications as days, e.g., ex 7 is the extract removed on the 7th day of incubation.

$A_{(t)}$ = % total nutrient/analyte where A is N.
ex x = An extract collected on a specific day (7, 14, 28, 56, 84, 140, or 180 days).
$AC_{(ex\ x)}$ = Analyte concentration (in mg/L) in extract x determined as in G above; where A is N.
$\%AR_{(ex\ x)}$ = % nutrient released during extraction x where A is N.
V = Volume (in mL) of respective extract collected.
W = Total unground test portion weight in g.

Calculations
(An example calculation is provided in Ref. 1)

$$\%AR_{(ex\ x)} = \left(\frac{AC_{(ex\ x),} \frac{mg}{L} x \ \frac{1\ g}{1000\ mg} x\ Vin\ ml/1000ml}{W, g\ x\ (A_t, \frac{g}{100\ g})} \right) x\ 100$$

H. Expression of Results

Results for each extraction are presented as cumulative percentage of total nutrient. Extraction 7 is considered water-soluble and not an SRF. However, slowly available water-soluble materials (low MW urea formaldehydes and methylene ureas) may be present. These materials can be analyzed directly from extract 7.

Graphing release plots.--Plot the cumulative % of analyte (nutrient) released on the y-axis versus days of extraction on the x-axis as in Figure **2015.15B**. (The example calculations are provided in ref. 1.)

Alternative B: Accelerated 74-Hour Extraction at 25–60°C

C. Apparatus

(a) Covered water bath capable of maintaining a temperature of up to 60°C for extended periods. Ensure the mean temperature in the system is 50.0, 55.0, or 60.0 ± 1.0°C by monitoring incoming and exit temperatures to the manifold at comparable locations. Before Extraction Nos. 2, 3, and 4 begin, it is necessary to preheat the bath several degrees (*see* X, Y, and Z in the *Extraction* section below) above the desired temperature to account for initial heat exchange and temperature equilibration with manifold and columns. Within 10 min, the bath should be stabilized at temperature X, Y, or Z.

(b) Reversible peristaltic pump capable of delivering 4.0 (±0.1) mL/min continuously for 54 h. Pump heads capable of using 16 to 40 tubes are used for an eight to 20 column apparatus, respectively (Lsmatc® No. 78006–00 by Cole-Parmer, Vernon Hills, IL).

(c) Extraction apparatus consisting of two parts (illustrated in Figures **2015.15C–F**). Example equipment as a parts list can be found in Appendix A of ref. 2 in the online *J. AOAC Int*.

(1) Vertical jacketed chromatography columns enclosing inner column of 2.5 × 30 cm (e.g., Ace Glass, Vineland, NJ; No. 5821–24, filter removed with Teflon® adapter No. 5838–51). PTFE rods (6 mm × 15 cm) should be used to avoid channeling of air or caking. All fittings needed to attach column, pump tubes, and transfer tubing (e.g., 2.4 mm id tubing). Standardize the length of tubing for each column (typically about 0.75 meter) and secure fittings.

(2) Constant temperature water circulation manifold and pump system capable of maintaining adequate (minimum 4 L/min) flow and stable temperature for each column. Insulation is typically required to maintain a stable temperature. Two inline, symmetrically placed thermometers (Figure **2015.15E**) are used to monitor temperature to input and outflow of manifolds. Attach roll clamps and flow monitors to column manifold tubing to ensure balanced flows and uniform temperatures.

(d) Solvent/extract reservoirs [500 mL volumetric flasks (e.g., Kimball Chase Life Science, Vinland, NJ; No. 28100–500)] with three-hole stoppers and properly placed rigid tubing attached transfer tubing and to pump (*see* Figure **2015.15E**). Make sure return tube remains approximately 2 cm from the bottom of the flask to prevent pickup of any precipitates.

(e) Detection equipment capable of analyzing liquids at moderate to high (100–10 000 mg/L) nutrient levels. Analysis falling below the LOD or LOQ should be noted.

(f) 250 mL graduated cylinders (8–20).

D. Reagents and Reference Materials

(a) *Extraction solution*.—0.2% citric acid [w/v, 40 g/20 L deionized (DI) water].

(b) Polyester fiber (available in fabric or craft stores).

(c) 250 mL amber HDPE (high density polyethylene) wide-mouth bottles for sample storage.

(d) Cupric sulfate solution stabilizer (20 g $CuSO_4$ $5H_2O$/L 1 + 1 HCl).

(e) Calibration standard, 500 mg N/L, matrix-matched to the liquid extracts for AOAC **993.13**.

(f) Matrix-matched (–7 + 9 mesh IBDU) internal reference material.

(f) 2% HCl/DI water solution for cleanup of equipment and tubing.

E. Sample Preparation of Granular Materials

(a) *Homogeneous or blended materials (coated N-P-Ks, granulations, or blends)*.—Reduce via gated riffle splitter (Jones Micro-Splitter SP-171X; Gilson Co., Inc., Lewis Center, OH) to 30.0 ± 1.0 g unground test portion. Place 3 (±0.2) g fiber 2–3 cm above the bottom of column (do not pack), and insert PTFE rod (ThermoFisher Scientific, Pittsburgh, PA). Using powder funnel, add test portion, and place 3 (±0.2) g fiber near top of column below O-ring, but not directly on top of test portion. Ensure no test portion or fibers foul O-ring seals.

(b) *Pellets, spikes, briquettes, etc*.—If larger than 2.5 cm, crack, crush, or break to yield pieces as large as possible that fit column (<2.5 cm). Use largest pieces equaling 30.0 ± 1.0 g, and weigh to ±.01 g. Place 3 (±0.2) g fiber approximately 2–3 cm above bottom of column (do not pack), insert polyethylene rod, add test portion, place 3 (±0.2) g fiber near top of column, but not on top of test portion. Ensure no fibers foul O-ring seals.

F. Extraction

Extraction sequence (examples in parenthesis)—

Day 1.—*Extraction 1*.—2 h at 25°C (e.g., Mon. 9:00 am– 11:00 am). *Extraction 2*.—2 h at 50°C. Begin 1 h following extraction 1 (e.g., Mon. 12:00 pm–2:00 pm). *Extraction 3*.—20 h at 55°C. Begin 1 h following extraction 2 (e.g., Mon. 3:00 pm– 11:00 am Tues.).

Day 2.—*Extraction 4*.—50 h at 60°C. Begin 1 h following extraction 3 (e.g., Tues. 12:00 pm–2:00 pm Thurs.).

Day 4.—*Extraction 5 (if needed).*—94 h at 60°C complete extraction 4 (e.g., Thurs. 3:00 pm). Begin extraction 5 one hour following extraction 4 (e.g., Thurs. 4:00 pm–2:00 pm Mon.).

Day 7.—Complete extraction 5; clean columns and system immediately.

(a) *Extraction 1.*—Adjust bath to maintain a temperature of 25 ± 1.0°C in columns and start circulation pump (Figure **2015.15E**). Add 475 mL extraction solution to each flask. Pump extraction solution and air from flasks to the bottom of the columns. Extract for exactly 2 h after solution reaches test portion. Swirl flask occasionally to mix solution during extraction. After 2 h, stop pump, reverse flow to top of column (Figure **2015.15F**); pump flows may be accelerated to hasten transfer process. Pump air for 1 min after liquid is emptied from column to ensure complete transfer of solution. Cool solution to 25.0°C, dilute to volume (500 mL) with extraction solution, and mix. Transfer exactly 250 mL extract to a storage bottle; add exactly 5.0 mL stabilizing solution. Extracts should be stored frozen or analyzed within 21 days. Remainder of test solution can be discarded. Extract 1 is ready for analysis.

(b) *Extraction 2.*—Immediately after completion of extraction No. 1, adjust bath to temperature X (the bath temperature needed to maintain 50.0 ± 1.0°C in columns). Drain manifold to preheat all manifold water. Start circulation to stabilize temperature in entire system 15 min before beginning extraction 2. Do not circulate water more than 5 min prior to extraction 2. Begin extraction 2 exactly 1 h after extraction 1 is complete. Add 475 mL extraction solution to flasks. Pump extraction solution and air from the flasks at 4 mL/min to the bottom of columns at predetermined time. Extract for exactly 2 h after solution first reaches samples. Swirl occasionally to mix extract solution during extraction. After 2 h, stop pump, reverse flow to top of columns, pumping solution back into flasks. Pump air for 1 min after all liquid is emptied to ensure maximum transfer of solution. Cool extract to 20°C, dilute to volume with solution, and mix. Using clean dry graduated cylinder, transfer exactly 250 mL of extract to amber HDPE bottles, and add exactly 5.0 mL of stabilizing solution. Extract is now ready for analysis. Keep all remaining 250 mL of solution in flasks to be used in next extraction. Add approximately 225 mL of freshly prepared extraction solution to flasks, bringing total volume to approximately 475 mL.

(c) *Extraction 3.*—Immediately after completion of extraction No. 2, adjust bath to temperature Y (the bath temperature needed to maintain 55.0 ± 1.0°C in columns). Drain manifold to preheat all manifold water. Start circulation to stabilize temperature in entire system 15 min before beginning extraction 2. Do not circulate water more than 5 min prior to extraction 3. Begin extraction 3 exactly 1 h after extraction 2 is complete. Remainder of extraction 3 is identical to No. 2 except extraction time is exactly 20 h.

(d) *Extraction 4.*—Immediately after completion of extraction No. 3 adjust bath to temperature Z (the system temperature needed to maintain 60.0 ± 1.0°C in columns). One hour after completion of extraction No. 3, begin extraction No. 4. Remainder of Extraction 4 is identical to No. 2 except extraction time is exactly 50 h.

(e) *Extraction 5 (if needed).*—One hour after completion of extraction No. 4, begin extraction No. 5. Extraction No. 5 is identical to No. 4 except extraction time is exactly 94 h.

Following removal of test portion, clean columns in place with a large brush. If there is buildup or

precipitation in columns or tubing, flush by circulating 2% HCl through system for 5 min. Follow with two 5 min DI water washes. If there is no buildup, water washes are sufficient. Allow columns to dry before placing new packing and samples in column for next run.

G. Analytical Determinations

Determine nutrients of interest (e.g., N, P, and K) on each of the extracts obtained.

(a) Determine total N using AOAC Method **993.13** (combustion) or AOAC Method **978.02** (modified comprehensive) or other equivalent, applicable methods validated in your laboratory. Utilize applicable method-matched reference material in each run. Utilize internal reference standard appropriate for the range of the sample extracts; typically 100, 1000, and 10000 mg N/L will cover the full range of N concentrations.

(**b**) Determine total phosphate (as P_2O_5) using AOAC Method **962.02** or AOAC Method **978.01** (1) or equivalent, applicable methods validated in your laboratory. Utilize applicable method-matched reference material in each run. Utilize internal reference standard appropriate for the range of the sample extracts; typically 100, 1000, and 10000 mg P_2O_5/L will cover the full range of P_2O_5 concentrations.

(**c**) Determine soluble potash (as K_2O) using AOAC Method **958.02** (STPB) or AOAC Method **983.02** (flame photometry; 1) or equivalent, applicable methods validated in your laboratory. Utilize applicable method-matched reference material in each run of samples. Utilize internal reference standard appropriate for the range of the sample extracts; typically 100, 1000, and 10 000 mg. K_2O/L will cover the full range of K_2O concentrations.

H. Expression of Results

Results for each extraction are presented as a cumulative percentage of total nutrient. Extraction No. 1 is water-soluble and not a slow-release fraction. However, slowly available water- soluble materials (low MW urea formaldehydes and methylene ureas) may be present. These materials can be analyzed directly from extraction No. 1 and expressed according to their definition.

I. Calculations and Graphing the Release Plot

Nomenclature for Extraction Calculations

$A_{(t)}$ = % Total nutrient/analyte determined as in the *Analytical Determinations* section above where A can be N, P, or K.

$AC_{(ex1)}$ = Concentration (in mg/L) of nutrient/analyte in extract No. 1, where A can be N, P, or K.

$\%AR_{(ex1)}$ = % Nutrient Released during extraction No. 1, where A can be N, P, or K.

$AC_{(ex2)}$ = Concentration (in mg/L) of nutrient in extract No. 2, where A can be N, P, or K.

$\%AR_{(ex2)}$ = % Nutrient released during extraction No. 2, where A can be N, P, or K.

$AC_{(ex3)}$ = Concentration (in mg/L) of nutrient in extraction No. 3, where A can be N, P, or K.

$\%AR_{(ex3)}$ = % Nutrient released during extraction No. 3, where A can be N, P, or K.

$AC_{(ex4)}$ = Concentration (in mg/L) of nutrient in extraction No. 4, where A can be N, P, or K.

$\%AR_{(ex4)}$ = % Nutrient released in extraction No. 3, where A can be N, P, or K.

$A_{(tr)}$ = % Total release of all extractions, where A can be N, P, or K.

$A_{(ne)}$ = % Controlled release (nutrient not extracted, calculated below), where A can be N, P, or K.

$A_{(cr)}$ = % Controlled release (nutrient released in Extractions 2–4), where A can be N, P, or K. W = Total unground test portion weight in g.

W = Total unground test portion weight in g.

%TAR = Total nutrient (analyte) released, where A can be N, P, or K.

WIN = Water insoluble nitrogen; 1.02 = dilution factor correction due to addition of the preservative = 255 mL/250 mL; V = volume, total, of respective extract, in L.

Calculations

Note: An example calculation can be found in Appendix B of ref. 2

$$\%AR_{(ex1)} = \left(\frac{AC_{(ex1),} \frac{mg}{L} x \frac{1\ g}{1000\ mg} x \left(\frac{0.255\ L}{0.250\ L}\right) x\ V, L}{W, g\ x\ (A_t, \frac{g}{100\ g})} \right) x\ 100$$

$$\%AR_{(ex2)} = \left(\frac{AC_{(ex2),} \frac{mg}{L} x \frac{1\ g}{1000\ mg} x \left(\frac{0.255\ L}{0.250\ L}\right) x\ V, L}{W, g\ x\ (A_t, \frac{g}{100\ g})} \right) x\ 100$$

$$\%AR_{(ex3)} = \left(\frac{\left(AC_{(ex3)} \frac{mg}{L} - \left(\frac{AC_{(ex2)} \frac{mg}{L}}{2} \right) \right) x \frac{1\ g}{1000\ mg} x \left(\frac{0.255\ L}{0.250\ L} \right) x\ V, L}{W, g\ x\ (A_t, \frac{g}{100\ g})} \right) x\ 100$$

$$\%AR_{(ex4)} = \left(\frac{\left(AC_{(ex4)} \frac{mg}{L} - \left(\frac{AC_{(ex3)} \frac{mg}{L}}{2} \right) \right) x \frac{1\ g}{1000\ mg} x \left(\frac{0.255\ L}{0.250\ L} \right) x\ V, L}{W, g\ x\ (A_t, \frac{g}{100\ g})} \right) x\ 100$$

$$\%TAR = \left(\%AR_{(ex1)}\right) + \left(\%AR_{(ex2)}\right) + \left(\%AR_{(ex3)}\right) + \left(\%AR_{(ex4)}\right)$$

$$\%WIN = (A_t\%) - \left((A_t\%) \left(\frac{\%AR_{(ex1)}}{100} \right) \right)$$

Graphing the Release Plot

Plot the cumulative % of analyte (nutrient) released on the y-axis versus the h of extraction on the x-axis. The release plot will be similar to Figure **2015.15B**, except for a change in the units on the x-axis from cumulative days of extraction to cumulative hours of extraction. An example graph can be found in Appendix B of ref. 2 in the online *J. AOAC Int*.

References

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J. AOAC Int. (future issue)

Posted: March 3, 2016

Table 2015.15A. Particle size analysis of Arredondo fine sand[a]

Mesh (US)	Opening, mm	Retained, %	Cumulative, %
5	4.000	0.0	0.0
10	2.000	0.4	0.4
20	0.850	1.2	1.6
40	0.425	12.4	14.0
100	0.150	68.7	82.7
200	0.075	14.5	97.2
-200		2.8	100.0

[a] Actual data, not specifications.

Table 2015.15B. 20/30 Particle size analysis of Topdress sand[a]

Mesh (US)	Opening, mm	Retained, %	Cumulative, %
5	4.000	0.0	0.0
10	2.000	0.0	0.0
20	0.850	11.6	11.6
40	0.425	34.2	45.9
100	0.150	51.9	97.8
200	0.075	2.2	99.9
-200		0.1	100.0

[a] Actual data, not specifications.

Figure 2015.15A. Incubation lysimeters.

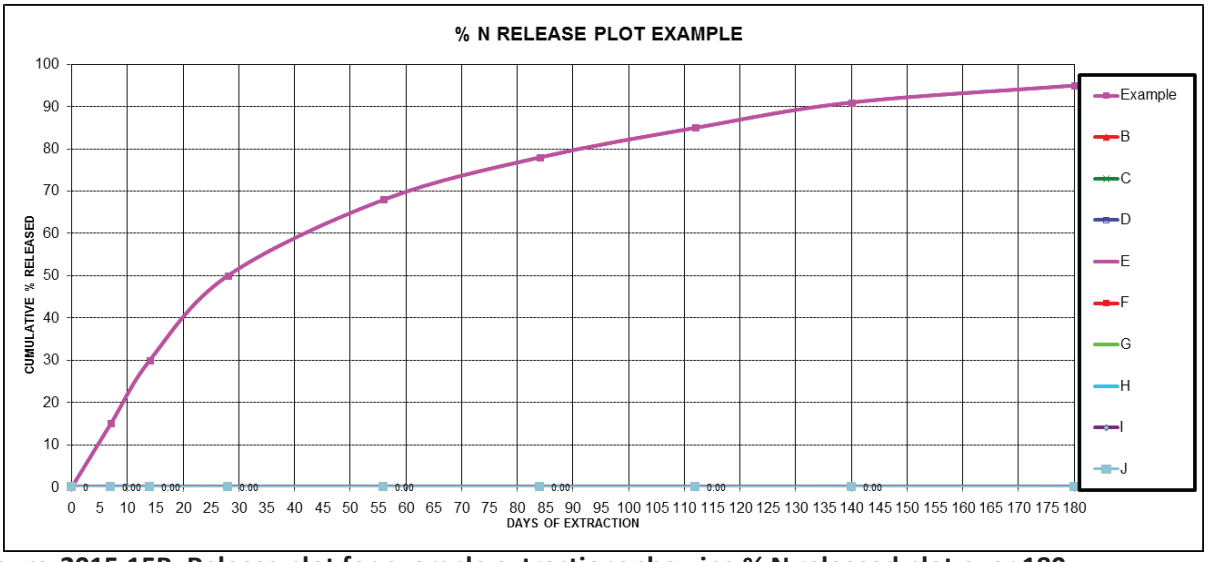

Figure 2015.15B. Release plot for example extractions showing % N released plot over 180 days.

Figure 2015.15C. Extraction apparatus with eight jacketed chromatography columns.

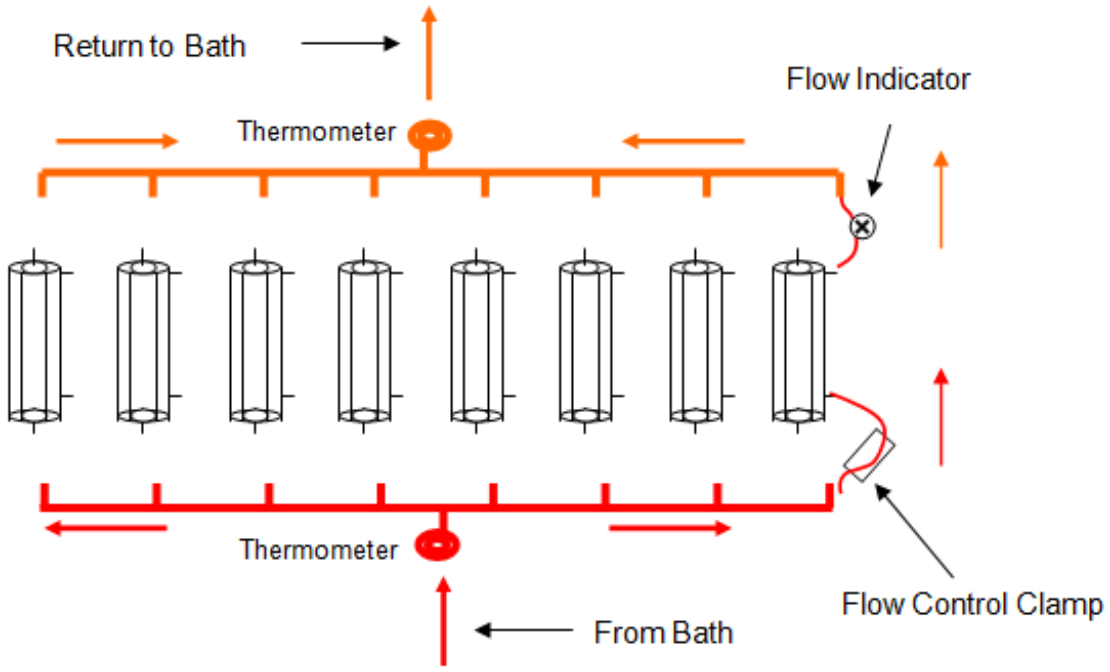

Figure 2015.15D. Schematic diagram of water manifold used in the extraction apparatus.

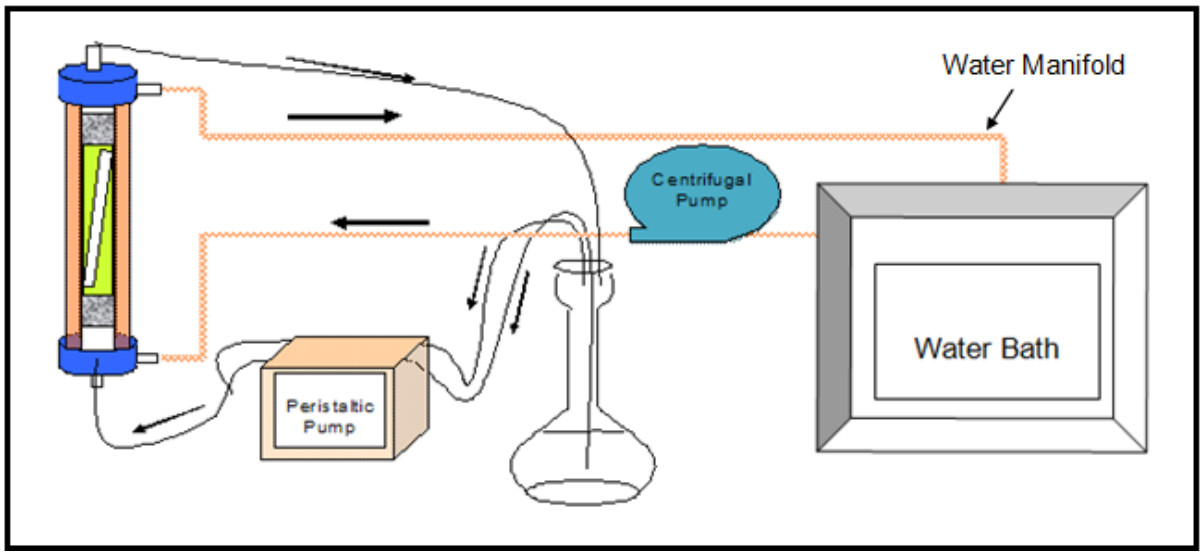

Figure 2015.15E. Schematic diagram of the extraction phase.

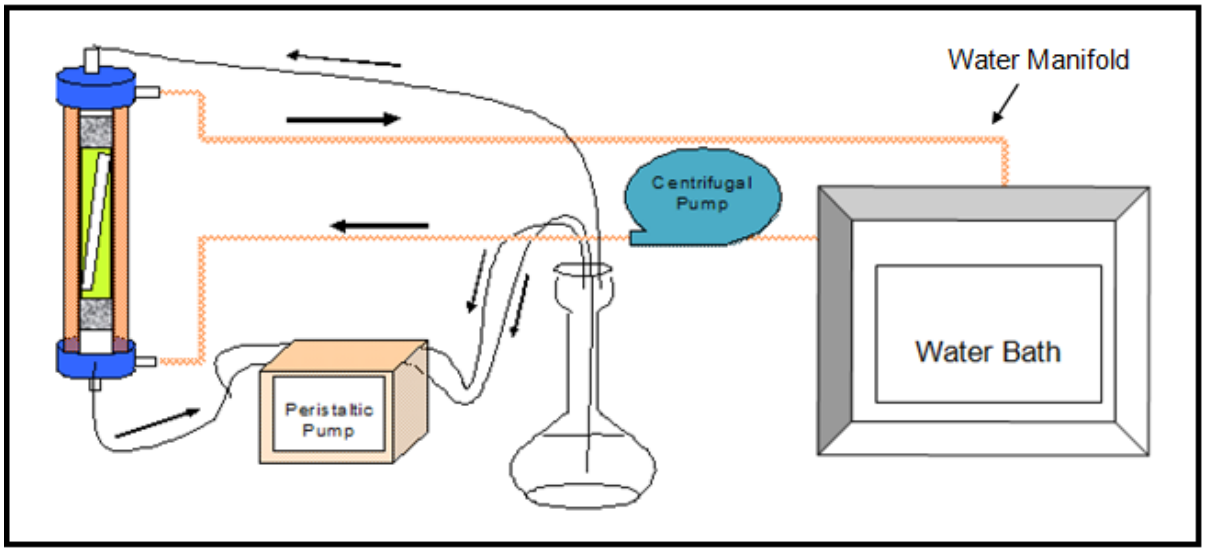

Figure 2015.15F. Schematic diagram of the collection phase.

AGRICULTURAL MATERIALS

Determination of Nitrogen, Phosphorus, and Potassium Release Rates of Slow- and Controlled-Release Fertilizers: Single-Laboratory Validation, First Action 2015.15

NANCY THIEX
Thiex Laboratory Solutions, Brookings, SD 57006

A previously validated method for the determination of nitrogen release patterns of slow- and controlled-release fertilizers (SRFs and CRFs, respectively) was submitted to the Expert Review Panel (ERP) for Fertilizers for consideration of First Action *Official Method*SM status. The ERP evaluated the single-laboratory validation results and recommended the method for First Action *Official Method* status and provided recommendations for achieving Final Action. The 180 day soil incubation-column leaching technique was demonstrated to be a robust and reliable method for characterizing N release patterns from SRFs and CRFs. The method was reproducible, and the results were only slightly affected by variations in environmental factors such as microbial activity, soil moisture, temperature, and texture. The release of P and K were also studied, but at fewer replications than for N. Optimization experiments on the accelerated 74 h extraction method indicated that temperature was the only factor found to substantially influence nutrient-release rates from the materials studied, and an optimized extraction profile was established as follows: 2 h at 25°C, 2 h at 50°C, 20 h at 55°C, and 50 h at 60°C.

Slow-release fertilizers (SRFs) and controlled-release fertilizers (CRFs) are designed to gradually release nutrients at rates that can more closely match nutrient demand by plants, while potentially reducing nutrient losses to the environment through leaching, volatilization, and/or runoff. Determining the nutrient-release patterns of SRFs and CRFs is essential in the agronomic evaluation of these materials (1). Although various field techniques had been used to investigate the agronomic effectiveness of SRFs and CRFs, a critical need existed for any laboratory method(s) that could be demonstrated to correlate with field data (1). In 1994, a Controlled-Release Fertilizer Task Force was established by the Association of American Plant Food Control Officials to address issues concerning the effective regulation and analysis of SRF and CRF materials (2, 3).

Received November 16, 2015. Accepted by RR January 13, 2016.
The method was approved by the Expert Review Panel on Fertilizers as First Action.

The Expert Review Panel on Fertilizers invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

Corresponding author's email: nancy.thiex@gmail.com

DOI: 10.5740/jaoacint.15-0294

The Fertilizer Methods Forum is a meeting for stakeholders to establish and prioritize method needs, communicate and discuss method validation results, organize and coordinate collaborative studies, and support volunteers involved in method development and validation. The Forum stakeholders placed a high priority on the development of any method(s) for nutrient release in SRFs and CRFs, and provided a forum for the evaluation of methods brought forth (4).

With the need for such methods well established by agronomists, industry, and regulatory communities, Carolina Medina undertook the validation of the Sartain et al. 180 day soil extraction to estimate nutrient release and the optimization and validation of a 4–7 day accelerated extraction method that resulted from the efforts of the Controlled-Release Fertilizer Task Force (5). The work was done as requirements for doctoral research at the University of Florida under the guidance of Thomas Obreza (University of Florida), Jerry Sartain (University of Florida), and William Hall (The Mosaic Co.). Medina et al.'s work was published as an evaluation of a 180 day soil extraction method to characterize N release patterns of SRFs and CRFs (1), an optimization and validation of an alternative accelerated 74 h extraction method (2), and a statistical correlation of the two extractions (6).

Method Optimization and Validation

180 Day Extraction

The effect of changes in soil/sand ratio, incubation temperature, and soil type on the 180 day soil incubation method to characterize the N release rates of various SRFs and CRFs were studied by Medina et al. (1) to establish the robustness of the method. These variables were tested on sulfur-coated urea, resin-coated NPK, polymer-sulfur-coated urea, reactive layer-coated urea, polyolefin-coated NPK, isobutylidenediurea, three types of ureaform, and biosolids.

The 180 day soil incubation-column leaching technique was demonstrated to be a robust and reliable method for characterizing N release patterns from SRFs and CRFs. The method was reproducible, and the results were only slightly affected by variations in environmental factors such as microbial activity, soil moisture, temperature, and texture. The release of P and K were also studied, but at fewer replications than for N.

Accelerated Extraction

Medina et al. (1) investigated the effect of extraction temperature, test portion mass, and extraction time on the ability of the accelerated extraction to estimate N, P, and K

release rates. Ruggedness testing was also performed using a fractional multifactorial design. Fertilizer materials used for the optimization experiments were polymer-coated urea, three types of ureaform, and two types of polymer-coated NPK. Fertilizer materials used for the ruggedness testing were polymer-coated urea, polymer-sulfur-coated urea, polyolefin-coated NPK, reactive layer-coated urea, and isobutylidenediurea,

Optimization experiments indicated that temperature was the only factor found to substantially influence nutrient-release rates from the materials studied. The optimal extraction temperature sequence that produced the most consistent and highly correlated N, P, and K release rates and showed no abnormal nutrient release due to coating deformation or fertilizer caking was determined to be:

Extraction 1.—2 h at 25°C
Extraction 2.—2 h at 50°C
Extraction 3.—20 h at 55°C
Extraction 4.—50 h at 60°C

Overall, the optimized method proved to be rugged for measuring N release rates of CRFs. The release of P and K were also studied, but at fewer replications than for N.

Method Summary

With the 180 day soil method, a fertilizer test portion is exposed to ambient temperature extractions with 0.01% citric acid in a biologically active sandy soil medium. Extractions are designed to extract and isolate nutrients becoming available over time (e.g., 7, 14, 28, 56, 140, and 180 days). Each extract is analyzed using an appropriate AOAC method (or comparable validated method) for the nutrient of interest. Cumulative nutrient released over time is calculated and release plots are graphed.

The alternative 74 h accelerated method provides an estimate of the 180 day method cumulative nutrient-release and nutrient-release plot within a time frame amenable to laboratory testing for manufacturing process control and regulatory testing to verify manufacturer label claims. Extractions are made with 0.2% citric acid and temperatures increased in a step-wise manner to accelerate the release of nutrients: 2 h at 25°C, 2 h at 50°C, 20 h at 55°C, 50 h at 60°C, and if needed, 94 h at 60°C.

AOAC Official Method 2015.15
Nitrogen, Phosphorus, and Potassium Release Rates of Slow- and Controlled-Release Fertilizers
First Action 2015

(Applicable for the determination of extractable N, P (as P_2O_5), and K (as K_2O) and cumulative N, P or K release in slow release fertilizers (SRFs) and controlled release fertilizers (CRFs).)

A. Principle

In Alternative A, a representative unground test portion is exposed to ambient temperature extractions of a solvent in a biologically active sandy soil medium. In Alternative B, a representative unground test portion is exposed to increasingly aggressive solvent temperature extractions. Extractions are designed to extract and isolate nutrients becoming available over time. Each extract is analyzed by AOAC procedures for the nutrient of interest (total N, P, and K). Along with analyses of total nutrients and reference materials, data are used to develop information specific to the cumulative percentage of nutrient released over time.

Alternative A: 180 Day Extraction at Ambient Temperature

B. Apparatus

(a) *Extraction columns*.—Extraction columns (incubation lysimeters; *see* Figure **2015.15A**) are constructed of PVC pipe (30 × 7.5 cm) fitted with a fiberglass mat in the bottom held in place by a 7.5 in. id PVC cap. The cap is fitted with a barbed plastic fitting, and vacuum tubing attached for leachate collection. A PVC cap is used on the top with no hole, but with a coating of stopcock grease to cap the lysimeter. All columns are supported on a wood frame.

(b) *Beaker*.—A 50 mL beaker is placed in the headspace of each incubation lysimeter.

(c) *Filtering flasks*.—Filtering flasks with a one-hole stopper are placed beneath the leaching columns and attached to the vacuum tubing. A pinch clamp is used to prevent leaks when filtration and leachate collection is complete.

(d) *Vacuum manifold*.—Vacuum manifold and tubing connecting each flask to a standard laboratory vacuum pump.

(e) *Riffle*.— gated or rotary.

C. Reagents and Reference Materials

(a) *Extraction solution*.—0.01% (w/v) citric acid [2 g/20 L deionized water (DI)] prepared from reagent-grade citric acid.

(b) *Ammonia trap solution*.—0.2 M H_2SO_4 solution.

(c) *Loamy, siliceous, hyperthermic, Grossarenic Paleudult soil*.—Arredondo fine sand. Particle size analysis is shown in Table **2015.15A**.

(d) *Uncoated quartz sand United States Golf Association Greens* (*USGA Mix)*.—Topdress sand (noncoated quartz), 20/30 silica sand. Available from Edgar Minerals Inc. (Edgar, FL) and Standard Sand and Silica Co. (Lynne, FL). Particle size analysis is shown in Table **2015.15B**.

(e) *Soil media*.—Mixture of 1710 g uncoated quartz sand, **C(d)**, and 90 g loamy siliceous, hyperthermic, Grossarenic Paleudult soil, **C(c)**, or similar type of local soil acting as a microbial inoculum.

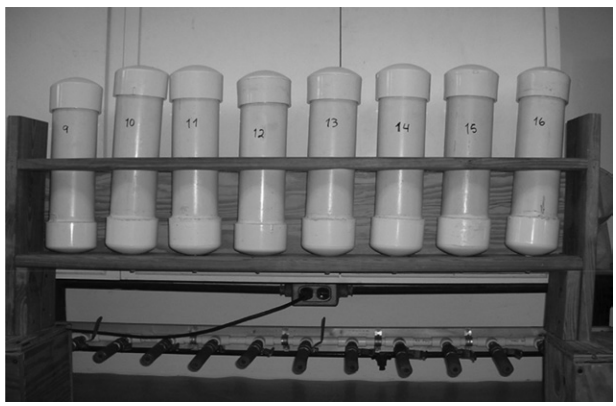

Figure 2015.15A. Incubation lysimeters.

Table 2015.15A. Particle size analysis of Arredondo fine sand[a]

Mesh (US)[b]	Opening, mm	Retained, %	Cumulative, %
5	4.000	0.0	0.0
10	2.000	0.4	0.4
20	0.850	1.2	1.6
40	0.425	12.4	14.0
100	0.150	68.7	82.7
200	0.075	14.5	97.2
−200		2.8	100.0

[a] Actual data, not specifications.

[b] United States Standard Mesh - ASTM E11:01.

D. Sample Preparation

(a) *For granular materials*.—Using a gated riffle splitter, reduce laboratory sample to yield an unground representative test portion containing approximately 450 mg of total N to mix thoroughly with the soil–sand mixture. If no N is present, a 3 (±0.1) g test portion should be used. Note: Quick release N must be limited to 600 mg N/test portion to prevent ammonia buildup in the column (thus preventing an active biological system); however, when doing so, replicates must be used to cumulatively measure at least 3.0 g total test portion mass and averaged to generate a single result. If soluble N is not limiting, 5–6 g of unground fertilizer should be used for the test portion.

(b) *For liquid materials*.—Assure the material is properly mixed and extract via pipet a representative test portion containing approximately 450 mg of total N. Mix thoroughly with the soil/sand mixture. Note: Quick release N must be limited to 600 mg N/test portion to prevent ammonia buildup in the column (thus preventing an active biological system); however, when doing so, replicates must be used to cumulatively measure at least 3.0 g total test portion mass and averaged to generate a single result . If soluble N is not limiting, 5–6 g of unground fertilizer should be used for the test portion.

E. Procedure

Test portions from each material to be tested are placed in incubation columns held at room temperature (20–25°C). The column preparation sequence is as follows: fiberglass mat, 100 g sand, then a mixture of remaining sand, soil, and test portion followed by placement of an acid trap. The sand–soil–test portion mixture is brought to 10% gravimetric moisture by adding 180 mL 0.01% citric acid. A 50 mL beaker containing 20 mL 0.2 M H_2SO_4 is placed in the headspace of the column as an ammonia trap. The solution in the ammonia trap is replaced and analyzed for NH_4-N by titration every 7 days. After 7, 14, 28, 56, 84, 112, 140, and 180 days, each column is leached at the same time of day with one pore volume (500 mL) 0.01% citric acid using a vacuum manifold. Vacuum is pulled for 2 min at 20–25" Hg vacuum (1.3 cfm) to ensure all free extraction solution is removed. Mix well and transfer to a 250 mL graduated cylinder. Record the leachate volume and remove aliquots to test for total N. In addition, measure the pH and electrical conductivity of the leachate. Retain the remaining leachate in reserve in case an additional or recheck analysis is required. Store in dark bottles and freeze if retained for more than 7 days. (*Note*: If no volatile N is detected in the ammonia trap during the first two sampling periods, the NH_4 trap can be removed and analysis for volatile N discontinued.)

Table 2015.15B. 20/30 particle size analysis of Topdress sand[a]

Mesh (US)[b]	Opening, mm	Retained, %	Cumulative, %
5	4.000	0.0	0.0
10	2.000	0.0	0.0
20	0.850	11.6	11.6
40	0.425	34.2	45.9
100	0.150	51.9	97.8
200	0.075	2.2	99.9
−200		0.1	100.0

[a] Actual data, not specifications.

[b] United States Standard Mesh - ASTM E11:01.

F. Analytical Determinations

(a) Determine total N in each of the extracts obtained using AOAC Method **993.13** (combustion), or **978.02** (modified comprehensive), or an equivalent applicable method validated in your laboratory. Use an applicable method-matched reference material in each run. Use at least three standards appropriate for the range of extract concentrations. Typically a combination of 10, 100, 1000, and 10 000 mg N/L cover the range of N in the extracts.

(b) Determine total phosphate (as P_2O_5) using AOAC Method **962.02** (gravimetric quinolinium) or AOAC Method **978.01** (automated spectrophotometric) or an equivalent applicable method validated in your laboratory. Use an applicable method-matched reference material in each run. Use internal reference standards appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg P_2O_5/L will cover the full range of P_2O_5 concentrations in the extracts.

(c) Determine soluble potash (as K_2O) using AOAC Method **958.02** sodium tetraphenylboron method or AOAC Method **983.02** (flame photometry) or an equivalent applicable method validated in your laboratory. Use an applicable method-matched reference material in each run of samples. Use internal reference standards appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg K_2O/L will cover the full range of K_2O concentrations.

Nomenclature for extraction calculation equations.—Time is measured in days and is expressed in the extract identifications as days; e.g., ex7 is the extract removed on the 7th day of incubation.

$$A_{(t)} = \%\ \text{Total nutrient/analyte}$$

where A can be N, P, or K.

ex *x* = An extract collected on a specific day (7, 14, 28, 56, 84, 140, or 180 days).

$$AC_{(\text{ex } x)} = \text{Analyte concentration (in mg/L)}$$

In extract *x* as determined in Section **F** above, where A can be N, P, or K.

$$\%AR_{(\text{ex } x)} = \%\ \text{Nutrient released during extraction } x$$

where A can be N, P, or K.

V = Volume (in mL) of respective extract collected.
W = Total unground test portion weight in g.
Calculations.—(An example calculation is provided in ref. 1.)

$$\%AR_{(ex\,x)} = \left(\frac{AC_{(ex\,x)}, \frac{mg}{L} \times \frac{1\ g}{1000\ mg} \times V, mL/1000\ mL}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

G. Expression of Results

Results for each extraction are presented as cumulative percentage of total nutrient. Extraction 1 (7 days) is considered water-soluble and not an SRF. However, slowly available water-soluble materials (low-MW urea formaldehydes and methylene ureas) may be present. These materials can be analyzed directly from Extract 1.

Graphing release plots.—Plot the cumulative % of analyte (nutrient) released on the *y*-axis versus days of extraction on the *x*-axis as in Figure **2015.15B**. (The example calculations are provided in ref. 1.)

Alternative B: Accelerated 74 h Extraction at 25–60°C

H. Apparatus

(a) Covered water bath capable of maintaining a temperature of up to 60°C for extended periods. Ensure the mean temperature in the system is 50.0, 55.0, or 60.0 ± 1.0°C by monitoring incoming and exit temperatures to the manifold at comparable locations. Before Extractions 2–4 begin, it is necessary to preheat the bath several degrees (*see Extraction* section below) above the desired temperature to account for initial heat exchange and temperature equilibration with manifold and columns. The bath should be stabilized at the desired temperature within 10 min.

(b) Reversible peristaltic pump capable of delivering 4.0 (±0.1) mL/min continuously for 54 h. Pump heads capable of using 16–40 tubes are used for an 8–20 column apparatus, respectively (Lsmatc® No. 78006–00; Cole-Parmer, Vernon Hills, IL).

(c) Extraction apparatus consisting of two parts (illustrated in Figures **2015.15C–F**). Example equipment with sources can be found as a parts list in Appendix A of ref. 2 available on the *J. AOAC Int*. web site.

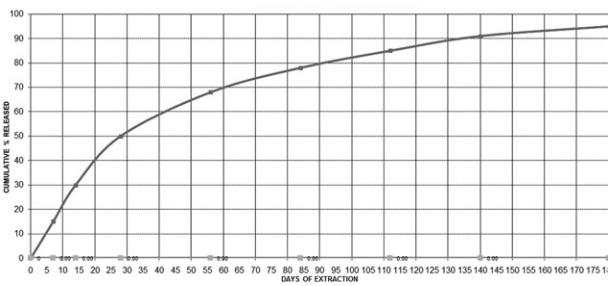

Figure 2015.15B. Example release plot showing % N released over 180 days.

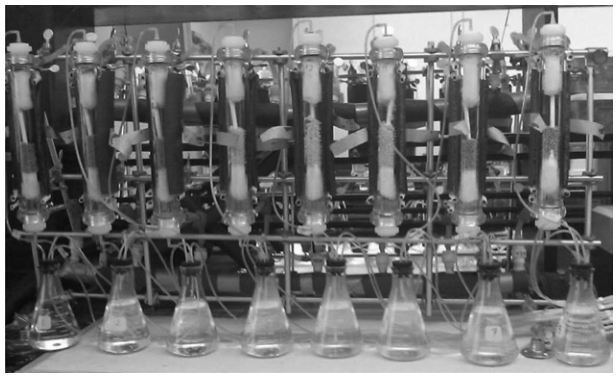

Figure 2015.15C. Extraction apparatus with eight jacketed chromatography columns.

(d) Vertical jacketed chromatography columns enclosing inner column of 2.5 × 30 cm (e.g., No. 5821–24, filter removed, with Teflon adapter No. 5838–51; Ace Glass, Vineland, NJ). PTFE rods (6 mm × 15 cm) should be used to avoid channeling of air or caking. Assure all fittings attaching column, pump tubes, and transfer tubing are secure to avoid leaks. Standardize the length of tubing for each column (typically about 75 cm). Example equipment with sources can be found as a parts list in Appendix A of ref. 2 available on the *J. AOAC Int*. web site.

(e) Constant temperature water circulation manifold and pump system capable of maintaining adequate (minimum 4 L/min) flow and stable temperature for each column. Insulation is typically required to maintain a stable temperature. Two inline, symmetrically placed thermometers (Figure **2015.15E**) are used to monitor temperature to input and outflow of manifolds. Attach roll clamps and flow monitors to column manifold tubing to ensure balanced flows and uniform temperatures. Example equipment with sources can be found as a parts list in Appendix A of ref. 2 available on the *J. AOAC Int*. web site.

(f) Solvent/extract reservoirs [500 mL volumetric flasks (e.g., Cat. No. 28100–500; Kimball Chase Life Science, Vinland, NJ)] with three-hole stoppers and properly placed rigid tubing attached to transfer tubing and to pump (*see* Figure **2015.15E**). Ensure return tube remains approximately 2 cm from the bottom of the flask to prevent pickup of any precipitates.

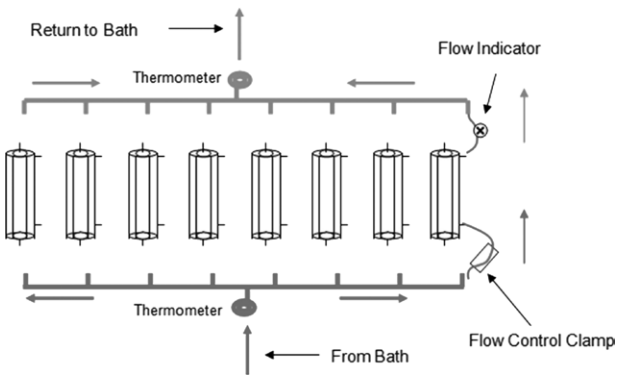

Figure 2015.15D. Schematic diagram of water manifold used in the extraction apparatus.

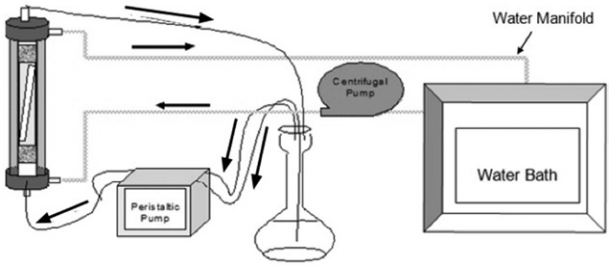

Figure 2015.15E. Schematic diagram of the extraction phase.

(g) Detection equipment capable of analyzing liquids at moderate to high (100–10 000 mg/L) nutrient levels. Analysis falling below the LOD or LOQ should be noted.

(h) 250 mL graduated cylinders (on an 8–20 column apparatus).

I. Reagents and Reference Materials

(a) *Extraction solution*.—0.2% Citric acid (w/v, 40 g/20 L DI water) prepared from reagent-grade citric acid.

(b) *Polyester fiber*.—A available in fabric or craft stores.

(c) *Wide mouth bottles*.—250 mL amber high-density polyethylene (HDPE) for sample storage.

(d) *0.08 M Cupric sulfate solution stabilizer*.—20 g $CuSO_4 \cdot 5\ H_2O$/L in 1 + 1 HCl.

(e) *Calibration standard*.—500 mg N/L, matrix-matched to the liquid extracts for AOAC **993.13**.

(f) *Matrix-matched internal reference material*.— –7 + 9 mesh IBDU.

(g) *HCl/DI water solution*.—2% for internal cleanup of equipment and tubing.

J. Sample Preparation

(a) *Homogeneous or blended materials (e.g., coated N-P-K fertilizers, granulations fertilzers, or blended fertilizers, etc.)*.—Reduce via rotary or gated riffle splitter (Jones Micro-Splitter SP-175X; Gilson Co., Inc., Lewis Center, OH) to 30.0 ± 1.0 g unground test portion. Place 3 (±0.2) g fiber [*see* Section **I(b)**] 2–3 cm above the bottom of column (do not pack), and insert PTFE rod (ThermoFisher Scientific, Pittsburgh, PA). Using powder funnel, add test portion and place 3 (±0.2) g fiber near the top of column below O-ring, but not directly on top of test portion. Ensure no test portion or fibers compromise O-ring seals. *Note*: A smaller test portion (e.g. 15g, but not less than 10g) may be used for homogeneous materials if column plugging occurs or if sample solubility constants dictate a lower sample solvent ratio to prevent solution saturation. If fine particles are escaping the column a syringe filter, type AP 20 glass fiber (2.0µm nominal pore size) in polyvinyl chloride (PVC) or polypropylene (PP) housing (e.g. EMD Millipore SLAP05010) may be added to the exit tubing just past the column to prevent material from being transferred to the reservoir.

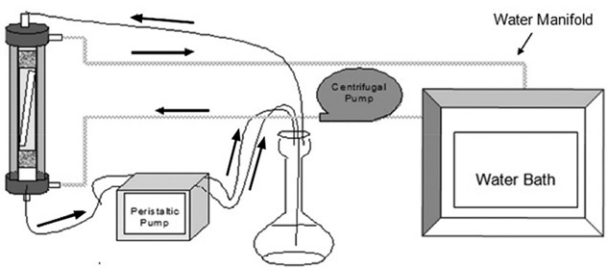

Figure 2015.15F. Schematic diagram of the collection phase.

(b) *Pellets, spikes, briquettes, etc*.—If larger than 2.5 cm, crack, crush, or break to yield pieces as large as possible that fit column (<2.5 cm). Use largest pieces equaling 30.0 ± 1.0 g and weigh to ±0.01 g. Place 3 (±0.2) g of fiber [*see* Section **I(b)**] approximately 2–3 cm above bottom of column (do not pack), insert polyethylene rod, add test portion, and place 3 (±0.2) g fiber near top of column, but not on top of test portion. Ensure no fibers compromise the O-ring seals.

(c) *For gelatinous or liquid materials*.—Assure the material is properly mixed and extract via pipet a representative test portion containing 30 ± 1.0g. Quantitatively add test portion to column, place 3g (± .2g) fiber 2–3 cm above the bottom of column (do not pack), insert PTFE rod. Add test portion, place 3g (± .2g) fiber near top of column below O ring, but not directly on top of test portion. Assure no test portion or fibers foul O ring seals. Note: A smaller test portion (e.g. 15g, but not less than 10g) may be used for homogeneous materials if column plugging occurs.

K. Extraction

Extraction sequence (examples in parenthesis).—Day 1.—Extraction 1.—2 h at 25°C (e.g., Monday 9:00 a.m.–11:00 a.m.). *Extraction 2*.—2 h at 50°C. Begin 1 h following Extraction 1 (e.g., Monday 12:00 p.m.–2:00 p.m.). *Extraction 3*.—20 h at 55°C. Begin 1 h following Extraction 2 (e.g., Monday 3:00 p.m.–11:00 am Tuesday).

Day 2.—Extraction 4.—50 h at 60°C. Begin 1 h following Extraction 3 (e.g., Tuesday 12:00 p.m.–2:00 p.m. Thursday).

Day 4.—Extraction 5 (if needed).—94 h at 60°C complete Extraction 4 (e.g., Thurs. 3:00 p.m.). Begin extraction 5, 1 h following Extraction 4 (e.g., Thursday 4:00 p.m.–2:00 p.m. Monday).

Day 7.—Complete Extraction 5; clean columns and system immediately.

(a) *Extraction 1*.—Adjust bath to maintain a temperature of 25 ± 1.0°C in columns and start circulation pump (Figure **2015.15E**). Add 475 mL extraction solution to each flask. Pump extraction solution and air from flasks to the bottom of the columns. Extract for exactly 2 h after solution reaches test portion. Swirl flask occasionally to mix solution during extraction. After 2 h, stop pump and reverse flow to top of column (Figure **2015.15F**); pump flows may be accelerated to hasten transfer process. Pump air for 1 min after liquid is emptied from column to ensure complete transfer of solution. Cool solution to 25.0°C, dilute to volume (500 mL) with 0.2% citric acid extraction solution, and mix. Transfer exactly 250 mL extract to a storage bottle; add exactly 5.0 mL stabilizing solution **I(d)**. Extracts should be stored frozen or analyzed within 21 days. Remainder of test solution can be discarded. Extract 1 is ready for analysis.

(b) *Extraction 2*.—Immediately after completion of Extraction 1, adjust bath to a temperature needed to maintain 50.0 ± 1.0°C in columns. Drain manifold(s) to preheat all

manifold water. Start circulation to stabilize temperature in entire system 15 min before beginning Extraction 2. Do not circulate water more than 5 min prior to Extraction 2. Begin Extraction 2 exactly 1 h after Extraction 1 is complete. Add 475 mL extraction solution to flasks. Pump extraction solution and air from the flasks at 4 mL/min to the bottom of columns at predetermined time. Extract for exactly 2 h after solution first reaches samples. Swirl occasionally to mix extract solution during extraction. After 2 h, stop pump and reverse flow to top of columns, pumping solution back into flasks. Pump air for 1 min after all liquid is emptied to ensure maximum transfer of solution. Cool extract to 20°C, dilute to volume with solution, and mix. Using a clean, dry graduated cylinder, transfer exactly 250 mL extract to amber HDPE bottles, and add exactly 5.0 mL stabilizing solution **I(d)**. Extract is now ready for analysis. Keep all remaining 250 mL of solution in flasks to be used in next extraction. Add approximately 225 mL freshly prepared extraction solution to flasks, bringing total volume to approximately 475 mL.

(c) *Extraction 3*.—Immediately after completion of Extraction 2, adjust bath to a temperature needed to maintain 55.0 ± 1.0°C in columns. Drain manifold(s) to preheat all manifold water. Start circulation to stabilize temperature in entire system 15 min before beginning Extraction 2. Do not circulate water more than 5 min prior to Extraction 3. Begin Extraction 3 exactly 1 h after Extraction 2 is complete. Remainder of Extraction 3 is identical to Extraction 2 except extraction time is exactly 20 h.

(d) *Extraction 4*.—Immediately after completion of Extraction 3, adjust bath to a temperature needed to maintain 60.0 ± 1.0°C in columns. 1 h after completion of Extraction 3, begin Extraction 4. Remainder of Extraction 4 is identical to Extraction 2 except extraction time is exactly 50 h.

(e) *Extraction 5 (if needed)*.—1 h after completion of Extraction 4, begin Extraction 5. Extraction 5 is identical to Extraction 4 except extraction time is exactly 94 h.

Following removal of the test portion, clean columns in place with a large brush. If there is buildup or precipitation in columns or tubing, flush by circulating 2% HCl through system for 5 min. Follow with two 5 min DI water washes. If there is no buildup, water washes are sufficient. Allow columns to dry before placing new packing and samples in column for next run.

L. Analytical Determinations

Determine nutrients of interest (e.g., N, P, and K) on each of the extracts obtained.

(a) Determine Total N using AOAC Method **993.13** (combustion) or AOAC Method **978.02** (modified comprehensive) or other equivalent applicable methods validated in your laboratory. Use an applicable method-matched reference material in each run. Use an internal reference standard appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg N/L will cover the full range of N concentrations.

(b) Determine total phosphate (as P_2O_5) using AOAC Method **962.02** or AOAC Method **978.01** or equivalent applicable methods validated in your laboratory. Use an applicable method-matched reference material in each run. Use an internal reference standard appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg P_2O_5/L will cover the full range of P_2O_5 concentrations.

(c) Determine soluble potash (as K_2O) using AOAC Method **958.02** (STPB) or AOAC Method **983.02** (flame photometry) or equivalent applicable methods validated in your laboratory. Use an applicable method-matched reference material in each run of samples. Use an internal reference standard appropriate for the range of the sample extracts; typically 10, 100, 1000, and 10 000 mg K_2O/L will cover the full range of K_2O concentrations.

M. Expression of Results

Results for each extraction are presented as a cumulative percentage of total nutrient. Extraction 1 is water-soluble and not a slow-release fraction. However, slowly available water-soluble materials (low-MW urea formaldehydes and methylene ureas) may be present. These materials can be analyzed directly from Extraction 1 and expressed according to their definition.

N. Calculations and Graphing the Release Plot

Nomenclature for extraction calculations.—$A_{(t)}$ = % Total nutrient/analyte determined as in the *Analytical Determinations* section above, where A can be N, P, or K.

$AC_{(ex1)}$ = Concentration (in mg/L) of nutrient/analyte in Extract 1, where A can be N, P, or K.

$\%AR_{(ex1)}$ = % Nutrient released during Extraction 1, where A can be N, P, or K.

$AC_{(ex2)}$ = Concentration (in mg/L) of nutrient in Extract 2, where A can be N, P, or K.

$\%AR_{(ex2)}$ = % Nutrient released during Extraction 2, where A can be N, P, or K.

$AC_{(ex3)}$ = Concentration (in mg/L) of nutrient in Extraction 3, where A can be N, P, or K.

$\%AR_{(ex3)}$ = % Nutrient released during Extraction 3, where A can be N, P, or K.

$AC_{(ex4)}$ = Concentration (in mg/L) of nutrient in Extraction 4, where A can be N, P, or K.

$\%AR_{(ex4)}$ = % Nutrient released in Extraction 4, where A can be N, P, or K.

$A_{(tr)}$ = % Total release of all extractions, where A can be N, P, or K.

$A_{(ne)}$ = % Controlled release (nutrient not extracted, calculated below), where A can be N, P, or K.

$A_{(cr)}$ = % Controlled release (nutrient released in Extractions 2–4), where A can be N, P, or K.

W = Total unground test portion weight in g.

%TAR = Total nutrient (analyte) released, where A can be N, P, or K.

WIN = Water-insoluble N.

1.02 = Dilution factor (due to addition of the preservative) = 255 mL/250 mL.

V = Total volume of respective extract in L.

Calculations.—Note: An example calculation can be found in Appendix B of ref. 2 available on the *J. AOAC Intl*. Web site.

$$\%AR_{(ex1)} = \left(\frac{AC_{(ex1)}, \frac{mg}{L} \times \frac{1\ g}{1000\ mg} \times \left(\frac{0.255\ L}{0.250\ L} \right) \times V, L}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

$$\%AR_{(ex2)} = \left(\frac{AC_{(ex2)}, \frac{mg}{L} \times \frac{1\ g}{1000\ mg} \times \left(\frac{0.255\ L}{0.250\ L} \right) \times V, L}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

$$\%AR_{(ex3)} = \left(\frac{\left(AC_{(ex3)}, \frac{mg}{L} - \left(\frac{AC_{(ex2)}, \frac{mg}{L}}{2} \right) \right) \times \frac{1\ g}{1000\ mg} \times \left(\frac{0.255\ L}{0.250\ L} \right) \times V, L}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

$$\%AR_{(ex4)} = \left(\frac{\left(AC_{(ex4)}, \frac{mg}{L} - \left(\frac{AC_{(ex3)}, \frac{mg}{L}}{2} \right) \right) \times \frac{1\ g}{1000\ mg} \times \left(\frac{0.255\ L}{0.250\ L} \right) \times V, L}{W, g \times \left(A_{(t)}, \frac{g}{100\ g} \right)} \right) \times 100$$

$$\%TAR = \left(\%AR_{(ex1)} \right) + \left(\%AR_{(ex2)} \right) + \left(\%AR_{(ex3)} \right) + \left(\%AR_{(ex4)} \right)$$

$$\%WIN = \left(A_{(t)}\% \right) - \left(\left(A_{(t)}\% \right) \left(\frac{\%AR_{(ex1)}}{100} \right) \right)$$

Graphing the release plot.—Plot the cumulative % of analyte (nutrient) released on the *y*-axis versus the time in hours of extraction on the *x*-axis. The release plot will be similar to Figure **2015.15B**, except for a change in the units on the *x*-axis from cumulative days of extraction to cumulative hours of extraction. An example graph can be found in Appendix B of ref. 2 available on the *J. AOAC Int*. web site.

Discussion

Medina et al.'s validated methods, including alternatives for a 180 day extraction and an accelerated extraction (1, 2), were submitted by William Hall to the AOAC Fertilizer Expert Review Panel (ERP), and adopted as First Action. Extensive method development and validation data supporting both alternatives as documented in Medina et al.'s studies (1, 2, 6) was further reviewed by the ERP. The validation was deemed thorough by the ERP, demonstrated that the method was scientifically sound, and confirmed that such a method is needed by the community.

Method reproducibility for both alternatives is proposed to be determined via collaborative study. The ERP asked that in addition to solid fertilizers, reproducibility data for liquid fertilizers be generated. The ERP also asked that the 180 day ambient method be clarified for its application to coated fertilizers (not including sulfur-coated) as reproducibility data are generated. It was noted that it might be difficult to obtain reproducibility data on the 180 day extraction within 2 years due to the long analysis time.

The collaborative studies will provide reproducibility data for N, P, K, and possibly other nutrients.

References

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(6) Medina, L.C., Sartain, J.B, Obreza, T.A, Hall, W.L., & Thiex, N.J. (2014) *J. AOAC Int.* **97**, 677–686. http://dx.doi.org/10.5740/jaoacint.13-004

AOAC Official Method 2015.16

Gluten in Processed and Nonprocessed Corn Products

Qualitative R5 Immunochromatographic Dip-Stick

First Action 2015

(RIDA®QUICK Gliadin is used for the qualitative analysis of gluten in nonprocessed and processed corn food products which are declared as "gluten-free.")

Caution: Ethanol is highly flammable and vapor; keep away from heat, hot surfaces, sparks, open flames and other ignition sources; do not smoke; keep container tightly closed; store in a well-ventilated place and keep cool. Cocktail solution contains 2-mercaptoethanol which is toxic; work under a chemical fume hood and avoid skin and eye contact and wear protective gloves and clothing (see MSDS).

See Tables **2015.16A** and **B** for results of method performance studies supporting acceptance of the method.

A. Principle

The dip stick consists of different zones (Figure **2015.16**). Analytes in the sample solution will be "chromatographed" above the 'maximum line' and react with the R5-antibody coupled to a red latex microsphere. The 'maximum line' indicates the user the maximal liquid level of the sample solution.

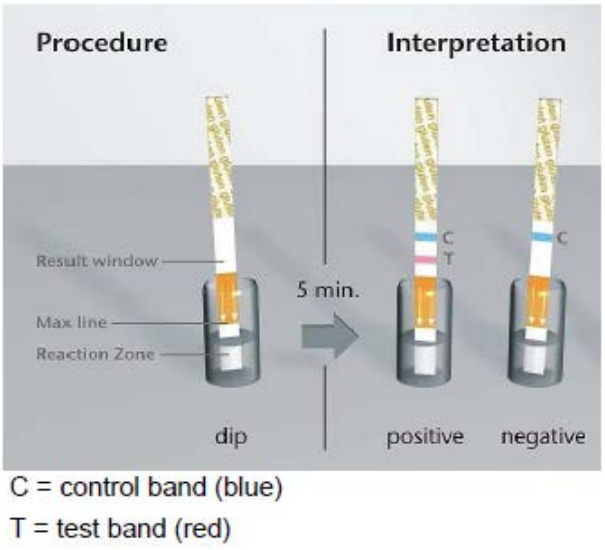

Figure 2015.16. Schematic presentation of the test principle and the subsequent interpretation of the possible results (invalid results not shown)

The 'result window' contains a small band of immobilized R5 antibody ('T'; red line after positive reaction) and a second line that turns blue when the reaction was valid. Results are read visually only. Generally, the higher the analyte level in the sample the stronger the red color of the test band will be (until a maximum of color is reached).

B. Apparatus

Apparatus specified here has been tested in the lab, equivalent apparatus may be used.

(a) Laboratory mincer/grinder, pestle and mortar, or Ultra-Turrax
(b) Scale
(c) Graduated cylinders (plastic or glass ware)
(d) Graduated pipettes
(e) Shaker (e.g. Roto Shaker Genie, Scientific Industries Inc.)
(f) Temperature controlled water bath 50 °C (e.g. GFL, Burgwedel, Germany)
(g) Centrifugal glass vials with a screw top
(h) Centrifuge (e.g. Minifuge RF, Kendro, Hanau, Germany)
(i) Paper filter
(j) Variable 20 – 200 µL and 200 – 1000 µL micropipettes

C. Reagents

Items (a-f) are available as a test kit (RIDA®QUICK Gliadin, R-Biopharm AG). All reagents are stable at least over a period of 18 months from date of manufacture at 2-8°C. Please refer to kit label for current expiration.

(a) Dip-sticks (25 x), sample diluent, test tubes (30 x), disposable pipettes (25 x), evaluation card (1 x).
(b) 25 x dip sticks in a tube
(c) 30 x empty test tubes
(d) 25 x disposable pipettes
(e) sample diluent (60 mL), ready to use, transparent capped bottle
(f) 1 x evaluation card

Necessary but not provided with the test kit:

(g) Distilled water
(h) Ethanol, 99% reagent grade
(i) Cocktail (patented); R7006 (R-Biopharm AG, Germany); ready to use
(j) Skim milk powder (food quality)

D. Standard Reference Material

Not currently available.

E. General Preparation

(a) *Sample diluent* --The sample diluent is ready to use. Bring the solution to room temperature (20-25 °C) before use. Make sure that the buffer is not contaminated with gluten during use.
(b) *60% aqueous ethanol* -- Add 150 mL ethanol to 100 mL distilled water and shake well
(c) *80% aqueous ethanol* -- Add 200 mL ethanol to 50 mL distilled water and shake well
(d) *Cocktail (patented)* -- The Cocktail is ready to use (see also C.)

F. General recommendation for sample preparation

(a) Store samples in a cold and dry room protected from light. Ensure that no cross-contamination takes place.
(b) Carry out the sample preparation in a room isolated from the dip-stick procedure
(c) Clean surfaces, glass vials, mincers and other equipment with 60 % ethanol (see E.) also after use for the next sample.
(d) Airborne cereal dust and used laboratory equipment may lead to gluten contamination of the assay. Therefore, wear gloves during the assay and before starting with the assay.
(e) If necessary, check for gluten contamination of reagents and equipment with the RIDA®QUICK Gliadin (Art. No. R7003).
(f) Keep in mind that solid samples can be inhomogeneous, therefore grind a representative part of the samples very well and homogenize before weighting.
(g) The sample extraction with ethanol should only be used for raw material that were surely not heated and not processed.
(h) All supernatants obtained after centrifugation can be stored in a tightly closed vial in the dark at room temperature (20-25 °C) up to four weeks.

G. Sample Preparation

Homogenize a representative amount of the sample (minimum 50 g; preferably 200 g).

(a) Nonprocessed samples

i. *Solid samples* -- Weigh 1 g of a representative, homogeneous sample in a vial and add 10 mL 60 % ethanol solution (see E.). For soy containing products add additionally 1 g of skim milk powder (see C.).

ii. Mix thoroughly for at least 30 s (vortex). Centrifuge the sample (2500 g at least) at room temperature (20-25 °C) for 10 min.; alternatively, let the sample settle down and/or filtrate. Dilute 50 µL supernatant with 500 µL sample diluent (see E.) in the test tubes (see C.) and subsequently proceed with H. (Determination).

(b) Processed samples

i. Weigh 0.25 g of a representative, homogeneous sample (pasty or solid) into a vial and add 2.5 mL Cocktail solution (see E.).

ii. Close the vial and mix well (vortex) to suspend the sample. Incubate the vial for 40 min at 50°C in the water bath. Let the sample cool down and add 7.5 mL 80% ethanol (see E.). Close the vial and shake the vial for 1 h up-side down or by a rotator at room temperature (20-25 °C). Centrifuge the sample (2500 g at least) at room temperature (20-25 °C) for 10 min.; alternatively, let the sample settle down and/or filtrate. Dilute 50 µL supernatant with 500 µL sample diluent (see E.) in the test tubes (see C.) and subsequently proceed with H.

H. General Recommendations for Good Test Performance

(a) This test should only be carried out by trained laboratory employees. The instructions for use must be strictly followed. No quality guarantee is accepted after expiry of the kit (see expiry label). Do not interchange individual reagents between kits of different lot numbers.

(b) Special attention should be directed to the interpretation of positive and negative outcomes (use of evaluation card and control samples).

(c) Bring the dip-sticks to room temperature (20-25 °C) before first use (after first use store at room temperature). The dip-sticks are very sensitive to humidity that could turn the test useless. For this reason keep the strips away from humidity.

(d) Use also gluten-free and gluten-containing samples as test controls (e.g. R7010 for ethanol extraction and R7012 for Cocktail extraction; both products are distributed by R-Biopharm AG, Germany). If the negative assay control sample is evaluated as positive then a contamination of the laboratory or laboratory equipment is likely.

(e) It is recommended to compare the extraction efficiency of ethanol with the Cocktail (patented) (R7006) in case of unknown samples.

I. Dip-Stick Testing

(a) Place the dip-stick vertically into the test tube filled with the diluted sample extract. The arrow on the dip-stick should point down (see also Figure **2015.16**). Do not immerse the dip-stick beyond the maximum line.

(b) Take out the stick after exactly 5 min (± 10 s) and evaluate the result using the evaluation card (see C.).

(c) For documentation and prolonged storage, the upper part of the dip-stick marked with “Gluten” together with the test bands should be cut off.

J. Dip-Stick Evaluation

(a) Positive result
If two colored bands (test band in red and control band in blue) are visible in the result window (see Figure **2015.16**) after 5 min, the sample is positive for gluten.

(b) Negative result
If only the blue control band is visible in the result window (see Figure **2015.16**) after 5 min, the sample is negative for gluten.

(c) Invalid result
If no bands occur after 5 min, the test is invalid and should be repeated using a new dip stick.

K. Result reporting

(a) Positive result
A non-processed sample contains more than 2.5 mg/kg gluten
A processed sample contains more than 4.0 mg/kg gluten

(b) Negative result
A nonprocessed sample contains less than 2.5 mg/kg gluten

A processed sample contains less than 4.0 mg/kg gluten

L. Result Interpretation

(a) The test strip has been developed for the detection of traces of gluten.
(b) A negative result does not necessarily indicate the absence of gluten as the gluten may be not homogenously distributed or the level of gluten in the product is below the limit of detection.
(c) The limit of detection is dependent on sample type and extraction efficiency.
(d) In case of a positive result, the RIDASCREEN® Gliadin (Art. No. R7001) should be used for quantification. This test kit is also AOAC-RI and AOAC-OMA (Official Method of Analysis, first action status) validated.

M. Criteria for Acceptance of a Result

(a) Accept results if quality control samples (R7012, R7013 or spiked samples) were evaluated correctly
(b) Appearance of test line and control line should be according to the evaluation card

Reference: *J. AOAC Int*. (future issue)

Posted: January 8, 2016

Table 2015.16A. Performance statistics for overall results using the R5 dip-stick after ethanol extraction

	Sample 1 (negative)		Sample 2 (low)		Sample 3 (medium)		Sample 4 (high)	
mg/kg gluten	1.76		4.84		11.0		18.8	
	positive	total	positive	total	positive	total	positive	total
Total (18 Labs)	2	180	177	180	178	180	180	180
POD		0.01		0.98		0.99		1.00
LCL		0.00		0.95		0.96		0.98
UCL		0.04		0.99		1.00		1.00
s(r)		0.10		0.13		0.10		0.00
s(R)		0.11		0.18		0.11		0.00

POD: probability of detection
LCL: lower limit of the confidence interval
UCL: upper limit of the confidence interval
s(r): repeatability standard deviation
s(R): reproducibility standard deviation

Table 2015.16B. Performance statistics for overall results using the R5 dip-stick after Cocktail extraction

	Sample 5 (negative)		Sample 6 (low)		Sample 7 (medium)		Sample 8 (high)	
mg/kg gluten	0.38		6.40		13.3		47.1	
	positive	total	positive	total	positive	total	positive	total
Total (17 Labs)	2	170	134	170	170	170	170	170
POD		0.01		0.79		1.00		1.00
LCL		0.00		0.72		0.98		0.98
UCL		0.04		0.84		1.00		1.00
s(r)		0.10		0.23		0.00		0.00
s(R)		0.11		0.42		0.00		0.00

POD: probability of detection
LCL: lower limit of the confidence interval
UCL: upper limit of the confidence interval
s(r): repeatability standard deviation
s(R): reproducibility standard deviation

FOOD COMPOSITION AND ADDITIVES

Determination of Gluten in Processed and Nonprocessed Corn Products by Qualitative R5 Immunochromatographic Dipstick: Collaborative Study, First Action 2015.16

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In September 2013, the AACC International (AACI) Protein Technical Committee (now Protein and Enzymes Technical Committee) initiated a collaborative study of a method for the qualitative analysis of intact gluten in processed and nonprocessed corn products, using an R5 immunochromatographic dipstick system. It was validated to demonstrate that potential gluten-free products contain gluten lower than the Codex threshold of 20 mg/kg gluten. The results of the collaborative test with 18 participants confirmed that the method is suitable to detect gluten contaminations that are clearly lower than the threshold. It is recommended that the method be accepted by AOAC as Official First Action.

With a population prevalence of 0.4 to 1.2% in Europe, North America, Australia, and the Middle East (1), celiac disease (CD) is considered one of the most common food intolerances. CD is an immune-mediated inflammatory disease of the upper small intestine in genetically predisposed individuals, and it is triggered by the ingestion of dietary gluten (2). In the context of CD, gluten is defined as a protein fraction from wheat, rye, barley, or their crossbred varieties and derivatives thereof, to which some persons are intolerant, and it is insoluble in water and 0.5 mol NaCl/L (3). Gluten is composed of prolamins that can be extracted by 40–70% ethanol and by alcohol-insoluble glutelins that can only be extracted under reducing and disaggregating conditions at elevated temperatures. The prolamins from wheat, rye, and barley are called gliadins, secalins, and hordeins, respectively, and the prolamin content of gluten is generally taken as 50% (3). The only known effective treatment for CD is a lifelong gluten-free diet, which is based on the avoidance of gluten-containing cereals and should contain less than 20 mg gluten/day to prevent a relapse of intestinal damage (4). To guarantee the safety of gluten-free products for CD patients, a threshold of 20 mg/kg gluten for gluten-free foods is required by the Codex Alimentarius and legislation, e.g., in the United States by the U.S. Food and Drug Administration, Department of Health and Human Services (5), and in Europe by the European Commission (6). Specific and sensitive analytical methods are therefore needed for food quality control. Immunochemical methods are currently recommended for the quantitative and qualitative determination of gluten in foods (3). Sandwich and competitive ELISA formats based on the R5 monoclonal antibody (7) were successfully validated as AACCI approved method 38-50.01 for intact gluten (8) and 38-55.01 for partially hydrolyzed gluten (9), respectively. Additionally, the R5 sandwich ELISA was laid down as a Codex Alimentarius Type I method for the analysis of gluten (10) and has been adopted by AOAC INTERNATIONAL as First Action *Official Method of Analysis*[SM] status **2012.01**. The R5 antibody raised against ω-secalins primarily recognizes the epitope QQPFP, which is present in gliadins, secalins, and hordeins and occurs in many peptides that are toxic or immunogenic for CD patients (11–13).

Immunochromatographic assays, usually available in dipstick or lateral-flow format, provide rapid, qualitative results indicating the presence or absence of the substance to be determined. The RIDA® QUICK Gliadin dipstick based on the R5 antibody is intended as a swab test of potentially contaminated surfaces and to check for gluten contamination of raw materials after ethanol extraction or a test of processed materials after Cocktail extraction (14).

An international collaborative study was set up to validate the R5 dipstick (RIDA QUICK Gliadin) for qualitative gluten detection in raw and processed corn food products as an AACCI-approved method. The study was carried out as collaboration

Received January 19, 2016. Accepted by SG March 16, 2016.
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The method was approved by the Expert Review Panel on Food Allergens.
The Expert Review Panel on Food Allergens invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit-for-purpose and are critical for gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or to methodfeedback@aoac.org.
[1] Presented at the AACC annual meeting in Providence, RI (October 7, 2014) and the Prolamin Working Group meeting in Nantes, France on September 25–27, 2014 by Katharina Sherf (nee Konitzer)
DOI: 10.5740/jaoacint.16-0017

between the Prolamin Working Group (PWG) and the AACCI. It was coordinated by Katharina Scherf (née Konitzer; German Research Center for Food Chemistry, vice-chair of the AACCI Protein Division, and co-chair of the AACCI Protein and Enzymes Technical Committee) and 18 participating laboratories.

Scope of the Method

RIDA QUICK Gliadin is used for the qualitative analysis of gluten in nonprocessed and processed corn food products that are declared "gluten-free." The immunochromatographic dipstick system detects intact prolamins from wheat (gliadins), rye (secalins), and barley (hordeins). The used R5 monoclonal antibody recognizes, among other things, the potentially immune-stimulatory sequence QQPFP, which occurs repeatedly in the prolamin proteins. Samples are extracted by 60% ethanol (nonprocessed food) or by Cocktail solution (processed food), are analyzed within 5 min, and are evaluated visually. The system was developed to detect gluten clearly below the threshold of 20 mg/kg and shows no high-dose hook effect.

Collaborative Study

Study Design

Following the AOAC guidelines, which are published as Appendix D (15) and Appendix N (16), an international collaborative study was set up to validate the R5 immunochromatographic dipstick (R-Biopharm RIDA QUICK Gliadin R7003) for qualitative gluten detection in processed and nonprocessed corn-containing foods as an AACCI-approved method. The study was carried out as a collaboration between the PWG and the AACCI. It was coordinated by Katharina Scherf (née Konitzer; German Research Center for Food Chemistry, vice-chair of the AACCI Protein Division, and co-chair of the AACCI Protein and Enzymes Technical Committee) in collaboration with Peter Koehler (German Research Center for Food Chemistry; chairman of the PWG and member of the Protein & Enzymes Technical Committee of AACCI) and Clyde Don (chair of the Protein & Enzymes Technical Committee of AACCI). Because this collaborative test is the first one following the new AOAC Appendix N, the study design was discussed and revised by Paul Wehling (AOAC statistician) in advance to ensure that the number of replicates and the number of concentration levels were sufficient. The collaborative test was split into two parts (A and B) to prevent mix-up of samples and procedures resulting from the different extractions. The total number of 40 samples per part is a compromise between the number of replicates and the number of concentration levels on the one hand, and the number of samples that a participant could manage within an acceptable time on the other hand. This compromise was partly compensated for by the high number of participants.

Collaborators

To qualify for participation in the collaborative test, all laboratories were required to have previous experience with immunological tests, such as ELISA, and to be familiar with the analytical procedure. Use of a separate room for the collaborative study was recommended because of the possibility of gluten contamination and the low detection limit. The laboratories were given 4 weeks each to perform the analyses for part A (April 1–30, 2014) and for part B (May 1–31, 2014). Eighteen laboratories (designated A to W) were chosen to participate: one each in Argentina, Austria, Belgium, Canada, Finland, Hungary, Ireland, Italy, Sweden, Switzerland, and the United Kingdom; three in Germany and four in the United States (*see* also *Acknowledgments*).

Samples and Sample Preparation

The main challenge for the validation of a qualitative method is the low amount of information per sample after analysis compared to a quantitative method. Therefore, a high number of replicate samples have to be analyzed. In general, the outline of the study followed the AOAC guidelines for validation of qualitative binary chemistry methods (Appendix N).

The following samples were prepared for part A of the collaborative study:

Sample 1.—Corn flour, containing gluten at 1.76 mg/kg.

Sample 2.—Corn flour, containing gluten at 4.84 mg/kg.

Sample 3.—Corn flour, containing gluten at 11.0 mg/kg.

Sample 4.—Corn flour, containing gluten at 18.8 mg/kg.

All concentrations were determined using the RIDASCREEN® Gliadin R7001 (R-Biopharm; AOAC First Action *Official Method of Analysis* status and Type I method according to the CODEX Alimentarius). Results are provided as mg/kg gluten by using the conversion factor of 2, which is mentioned in Codex Standard 118-1979. Sample 1 was a "gluten-free" corn flour with a gluten concentration below the LOQ (5.0 mg/kg gluten) of the method. Nevertheless, to obtain an idea of the contamination level, values were extrapolated from the calibration curve of the quantitative sandwich assay (8) and showed that a very low contamination of gluten was present (1.76 mg/kg). The corn flour samples 2–4 were prepared by mixing a naturally contaminated corn flour sample with the "gluten-free" corn flour sample 1.

The following samples were prepared for part B of the collaborative study:

Sample 5.—Cookie (processed), containing gluten at 0.38 mg/kg.

Sample 6.—Corn snack (processed), containing gluten at 6.40 mg/kg.

Sample 7.—Corn snack (processed), containing gluten at 13.3 mg/kg.

Sample 8.—Corn snack (processed), containing gluten at 47.2 mg/kg.

The processed snack samples 6–8 were prepared by mixing a snack sample (spiked at 100 mg gluten/kg before processing) with a "gluten-free" snack sample. Both samples were already used in the collaborative test of the RIDASCREEN Gliadin (R7001), which was published including a description of the preparation of these samples (8). Because the "gluten-free" snack sample showed a low contamination level during the collaborative test in 2012, a commercial gluten-free cookie (sample 5) was used instead as a "zero-gluten" sample for the study of the RIDA QUICK Gliadin dipstick. The value for sample 5 was extrapolated from the calibration curve (8).

All materials were prepared by grinding to ensure all materials passed a 40-mesh screen and were combined methodically to ensure homogeneity. The complete sample was mixed for 2 h, sieved through a 40-mesh screen, and then mixed again. Samples were packaged for delivery into foil pouches at an amount of 0.7 g for processed samples and 2.8 g for nonprocessed samples.

Homogeneity of Samples

Homogeneity was tested using the R5 sandwich ELISA (RIDASCREEN Gliadin, R-Biopharm, R7001). The determination of homogeneity was performed according to the IUPAC recommendations for proficiency tests (17). The SD (s_p) was derived from the Horwitz equation to calculate a deviation that is dependent on the concentration. In brief, 10 bags were randomly chosen and two subsamples were taken from each bag. After analyzing all samples (in sum 20), the calculation was performed as described in the IUPAC guideline. All samples turned out to be homogenous according to the guidelines.

Presentation of Samples to Laboratories

Following the collaborative test guidelines of AOAC and in accordance with AOAC Appendix N, 10 blinded replicates for each sample were provided to each participating laboratory. As already stated, the number of replicates is a compromise between statistics and the workload for each participant.

The samples were marked with a laboratory-specific letter (A–W), an "E" for ethanol extraction or a "C" for Cocktail extraction, and a randomized number from 1 to 40. Each laboratory obtained its own coding (different randomized numbers for each laboratory).

Method and Qualitative Evaluation

The method was written in AACCI style and was provided to each laboratory with the instructions to follow the method as written with no deviations. All results obtained by visual inspection had to be recorded in a ready-to-use Excel sheet. The final data from the laboratories were sent to the study coordinator.

Before analyzing the blind-coded samples, each participant was asked to perform checks for contamination and to become familiar with the test method. The latter was necessary because the qualitative nature of the obtained result made a later check for sample mix-up or improper testing very difficult.

Checks for Contamination

Possible sources of contamination during sample preparation and the test evaluation include the laboratory equipment, such as containers and surfaces, the Cocktail solution, the 60 or 80% ethanol solution, and the dilution buffer. To check for these possible sources, the participants were asked to perform two experiments before starting to analyze the blind-coded samples. (*1*) The dilution buffer (containing Cocktail and/or ethanol) was checked for gluten contamination. (*2*) A swab test of the laboratory bench across a sampling area of about 10 × 10 cm using the dipstick was performed. If both tests were negative, the participants were allowed to proceed with the analysis. No participant reported a positive result to the study coordinator.

Training and Familiarization with the Test

Because of the fact that outlier detection after performing the analysis is complicated, the participants obtained a training video and two sets of assay controls with known concentrations to check their own performance. One set was for part A (available as R7010; R-Biopharm) and the other one was for part B (available as R7012; R-Biopharm). To standardize the results, the test kit manufacturer inserted an evaluation card in the test kit.

Finally, each blind-coded sample was extracted once and was analyzed according to the test kit instruction. In total, 80 samples had to be analyzed by each laboratory. Each sample had to be marked positive or negative or invalid. In case of an invalid result (missing control line or incomplete target line), retesting of the sample was requested. No participant reported an invalid result to the study coordinator.

Method

Gluten is measured in food containing wheat, rye, and barley. Gluten is detected in processed and nonprocessed corn products by qualitative R5 immunochromatographic dipstick.

AOAC Official Method 2015.16
Gluten in Processed and Nonprocessed Corn Products
Qualitative R5 Immunochromatographic Dipstick
First Action 2015

[Presented by Katharina Scherf (née Konitzer) at the American Association of Cereal Chemists (AACC) annual meeting, Providence, RI, October 7, 2014, and the Prolamin Working Group meeting, Nantes, France, September 25–27, 2014.]

(Applicable for RIDA QUICK Gliadin for the qualitative analysis of gluten in nonprocessed and processed corn food products that are declared as "gluten-free.")

Caution: Ethanol is a highly flammable vapor. Keep away from heat, hot surfaces, sparks, open flames, and other ignition sources. Do not smoke. Keep container tightly closed. Store in a well-ventilated place and keep cool. For Cocktail solution containing 2-mercaptoethanol, which is toxic, work under a chemical fume hood, avoid skin and eye contact, and wear protective gloves and clothing (*see* MSDS, attached as separate documents or delivered by the manufacturer in the case of ethanol).

A. Principle

The dipstick consists of different zones (Figure **2015.16**). Analytes in the sample solution will be "chromatographed" above the "maximum line" and react with the R5-antibody coupled to a red latex microsphere. The "maximum line" indicates to the user the maximal liquid level of the sample solution.

The "result window" contains a small band of immobilized R5 antibody ("T"; red line after positive reaction) and a second line that turns blue when the reaction is valid. Results are read visually only. Generally, the higher the analyte level in the sample the stronger the red color of the test band (until a maximum of color is reached).

B. Apparatus

Apparatus specified here has been tested in the laboratory; equivalent apparatus may be used.

(a) *Laboratory mincer/grinder, pestle and mortar, or Ultra-Turrax*.

(b) *Scale*.

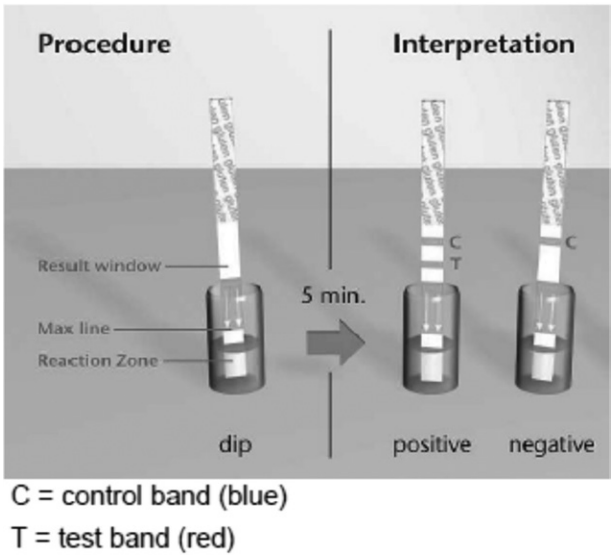

Figure 2015.16. Schematic presentation of the test principle and the subsequent interpretation of the possible results (invalid results not shown).

(c) *Graduated cylinders (plastic- or glassware)*.
(d) *Graduated pipets*.
(e) *Shaker*.—e.g., Roto Shaker Genie, Scientific Industries Inc.
(f) *Water bath*.—Temperature controlled 50°C (e.g., GFL, Burgwedel, Germany).
(g) *Centrifugal glass vials with a screw top*.
(h) *Centrifuge*.—e.g., Minifuge RF, Kendro, Hanau, Germany.
(i) *Paper filter*.
(j) *Micropipets*.—Variable 20–200 µL and 200–1000 µL.

C. Reagents

Items **(a–f)** are available as a test kit (RIDA QUICK Gliadin, R-Biopharm AG). All reagents are stable at least throughout a period of 18 months from date of manufacture at 2–8°C. Please refer to kit label for current expiration.
(a) *25 × dipsticks in a tube*.
(b) *30 × empty test tubes*.
(c) *25 × disposable pipets*.
(d) *Sample diluent (60 mL), ready to use, transparent capped bottle*.
(e) *1× evaluation card*.

Necessary but not provided with the test kit:
(f) *Distilled water*.
(g) *Ethanol, 99% reagent grade*.
(h) *Cocktail (patented)*.—R7006 (R-Biopharm AG, Germany); ready to use.
(i) *Skim milk powder (food quality)*.

D. Standard Reference Material

Not currently available

E. General Preparation

(a) *Sample diluent*.—The sample diluent is ready to use. Bring the solution to room temperature (20–25°C) before use. Make sure that the buffer is not contaminated with gluten during use.
(b) *60% Aqueous ethanol*.—Add 150 mL ethanol to 100 mL distilled water and shake well.
(c) *80% Aqueous ethanol*.—Add 200 mL ethanol to 50 mL distilled water and shake well.
(d) *Cocktail (patented)*.—The Cocktail is ready to use (C).

F. General Recommendation for Sample Preparation

(a) Store samples in a cold and dry room protected from light. Ensure that no cross-contamination takes place.
(b) Carry out the sample preparation in a room isolated from the dipstick procedure.
(c) Clean surfaces, glass vials, mincers, and other equipment with 60% ethanol (E) and also after use for the next sample.
(d) Airborne cereal dust and used laboratory equipment may lead to gluten contamination of the assay. Therefore, wear gloves during the assay and before starting with the assay.
(e) If necessary, check for gluten contamination of reagents and equipment with the RIDA QUICK Gliadin (Art. No. R7003).
(f) Keep in mind that solid samples can be inhomogeneous, therefore grind a representative part of the samples very well and homogenize before weighing.
(g) The sample extraction with ethanol should only be used for raw material that were surely not heated and not processed.
(h) All supernatants obtained after centrifugation can be stored in a tightly closed vial in the dark at room temperature (20–25°C) for up to 4 weeks.

G. Sample Preparation

Homogenize a representative amount of the sample (minimum 50 g; preferably 200 g).
(a) *Nonprocessed samples*.—(*1*) *Solid samples*.—Weigh 1 g of a representative, homogeneous sample in a vial and add 10 mL 60% ethanol solution (E). For soy-containing products additionally add 1 g skim milk powder (C).
(*2*) Mix thoroughly for at least 30 s (vortex). Centrifuge the sample (2500 *g* at least) at room temperature (20–25°C) for 10 min; alternatively, let the sample settle down and/or filtrate. Dilute 50 µL supernatant with 500 µL sample diluent (E) in the test tubes (C) and subsequently proceed with H.
(b) *Processed samples*.—(*1*) Weigh 0.25 g of a representative, homogeneous sample (pasty or solid) into a vial and add 2.5 mL Cocktail solution (E).
(*2*) Close the vial and mix well (vortex) to suspend the sample. Incubate the vial for 40 min at 50°C in the water bath. Let the sample cool and add 7.5 mL 80% ethanol (E). Close the vial and shake for 1 h upside down or by a rotator at room temperature (20–25°C). Centrifuge the sample (2500 *g* at least) at room temperature (20–25°C) for 10 min; alternatively, let the sample settle down and/or filtrate. Dilute 50 µL supernatant with 500 µL sample diluent (E) in the test tubes (C) and subsequently proceed with **H**.

H. General Recommendations for Good Test Performance

(a) This test should only be carried out by trained laboratory employees. The instructions for use must be strictly followed.

No quality guarantee is accepted after expiry of the kit (*see* expiry label). Do not interchange individual reagents between kits of different lot numbers.

(b) Special attention should be directed to the interpretation of positive and negative outcomes (use of evaluation card and control samples).

(c) Bring the dipsticks to room temperature (20–25°C) before first use (after first use, store at room temperature). The dipsticks are very sensitive to humidity, which could turn the test useless. For this reason, keep the strips away from humidity.

(d) Use also gluten-free and gluten-containing samples as test controls (e.g., R7010 for ethanol extraction and R7012 for Cocktail extraction; both products are distributed by R-Biopharm AG, Germany). If the negative assay control sample is evaluated as positive, then a contamination of the laboratory or laboratory equipment is likely.

(e) It is recommended to compare the extraction efficiency of ethanol with the Cocktail (patented; R7006) in the case of unknown samples.

I. Dipstick Testing

(a) Place the dipstick vertically into the test tube filled with the diluted sample extract. The arrow on the dipstick should point down (*see* also Figure **2015.16**). Do not immerse the dipstick beyond the maximum line.

(b) Take out the stick after exactly 5 min (±10 s) and evaluate the result using the evaluation card (C).

(c) For documentation and prolonged storage, the upper part of the dipstick marked with "Gluten," together with the test bands, should be cut off.

J. Dipstick Evaluation

(a) *Positive result*.—If two colored bands (test band in red and control band in blue) are visible in the result window (*see* Figure **2015.16**) after 5 min, the sample is positive for gluten.

(b) *Negative result*.—If only the blue control band is visible in the result window (*see* Figure **2015.16**) after 5 min, the sample is negative for gluten.

(c) *Invalid result*.—If no bands occur after 5 min, the test is invalid and should be repeated using a new dipstick.

K. Result Reporting

(a) *Positive result*.—A nonprocessed sample contains more than 5.0 mg/kg gluten. A processed sample contains more than 8.0 mg/kg gluten.

(b) *Negative result*.—A nonprocessed sample contains less than 5.0 mg/kg gluten. A processed sample contains less than 8.0 mg/kg gluten.

L. Result Interpretation

(a) The test strip has been developed for the detection of traces of gluten.

(b) A negative result does not necessarily indicate the absence of gluten as the gluten may not be homogenously distributed or the level of gluten in the product is below the LOD.

(c) The LOD is dependent on sample type and extraction efficiency.

(d) In case of a positive result, the RIDASCREEN Gliadin (Art. No. R7001) should be used for quantification. This test kit is also AOAC Research Institute and AOAC First Action *Official Method of Analysis* status validated.

M. Criteria for Acceptance of a Result

(a) Accept results if quality control samples (R7012, R7013, or spiked samples) are evaluated correctly.

(b) Appearance of test line and control line should be according to the evaluation card.

Results and Discussion

Collaborative Study Results

All participants reported to the study director that no contamination occurred in their laboratories and that all control samples were evaluated in the expected way.

The results for each sample and each laboratory are shown in Table 1 (ethanol extraction) and Table 2 (Cocktail extraction). Every laboratory analyzed 10 replicates for each concentration. Especially for the ethanol extraction, the results were uniform and 14 of 18 laboratories showed no false positives or false negatives. From the remaining four laboratories, only one laboratory assigned 2 of 10 blank samples as false positives. The other three laboratories found one false negative for the low concentration and only one laboratory found two false negatives

Table 1. Numbers of positive samples detected using the R5 dipstick after ethanol extraction[a]

		Sample 1 (negative)	Sample 2 (low)	Sample 3 (medium)	Sample 4 (high)
Gluten, mg/kg		1.76	4.84	11.0	18.8
Laboratory code	Total	Positive	Positive	Positive	Positive
A	10	0	10	10	10
B	10	0	10	10	10
D	10	0	10	10	10
E	10	0	10	10	10
F	10	0	10	10	10
G	10	0	10	10	10
H	10	0	10	10	10
I	10	0	9	10	10
L	10	0	10	10	10
M	10	0	9	8	10
N	10	0	10	10	10
O	10	0	10	10	10
P	10	0	10	10	10
R	10	0	10	10	10
S	10	0	9	10	10
T	10	0	10	10	10
U	10	0	10	10	10
W	10	2	10	10	10

[a] Data by each of the 18 participating laboratories; each laboratory obtained 10 blinded replicates for each concentration level.

Table 2. Numbers of positive samples detected using the R5 dipstick after Cocktail extraction[a]

		Sample 5 (negative)	Sample 6 (low)	Sample 7 (medium)	Sample 8 (high)
Gluten, mg/kg		0.38	6.4	13.3	47.1
Laboratory code	Total	Positive	Positive	Positive	Positive
A	10	2	7	10	10
B[b]	10	1	10	10	9
D	10	0	9	10	10
E	10	0	1	10	10
F	10	0	10	10	10
G	10	0	10	10	10
H	10	0	10	10	10
I	10	0	9	10	10
L	10	0	8	10	10
M	10	0	10	10	10
N	10	0	10	10	10
O	10	0	10	10	10
P	10	0	10	10	10
R	10	0	10	10	10
S	10	0	0	10	10
T	10	0	9	10	10
U	10	0	1	10	10
W	10	0	10	10	10

[a] Data by each of the 18 participating laboratories; each laboratory obtained 10 blinded replicates for each concentration level.

[b] Data set of Laboratory B was not included in the statistical calculation because two samples were apparently exchanged.

for the medium concentrated sample. It should be kept in mind that the concentration of the blank sample was clearly below the LOQ of the quantitative ELISA method, but still detectable. At these low concentrations, an inhomogeneity is not impossible and, therefore, a few false positives (2 of 180 samples) could be expected from this viewpoint.

The Cocktail extraction procedure ends up with a 4-fold higher dilution compared to the ethanol extraction. Therefore it was not surprising that the low concentrated sample showed a higher variation compared to the ethanol extraction. Laboratory B had to be excluded because it was obvious from the raw data (Excel sheet sent to the study coordinator) that a blank sample had been mixed up with a sample containing the high concentration. Nevertheless, 9 of 17 laboratories reported no false-negative or false-positive results. Only one laboratory found false-positive results. In total, 2 of 170 samples were detected as false positive. This rate is the same as for the ethanol extraction method. It is interesting to see that for the low-concentrated sample (6.4 mg/kg), laboratories could be separated into two groups reporting either 70 up to 100% correct detection or 0 to 10% correct results. It seems that the visual inspection results in a clear individual cut-off "color" for a positive sample and not—as speculated from a hypothetical point of view—a variation within the fractional range. In conclusion, it will be difficult to find or prepare a sample within the fractional range as requested by AOAC Appendix N.

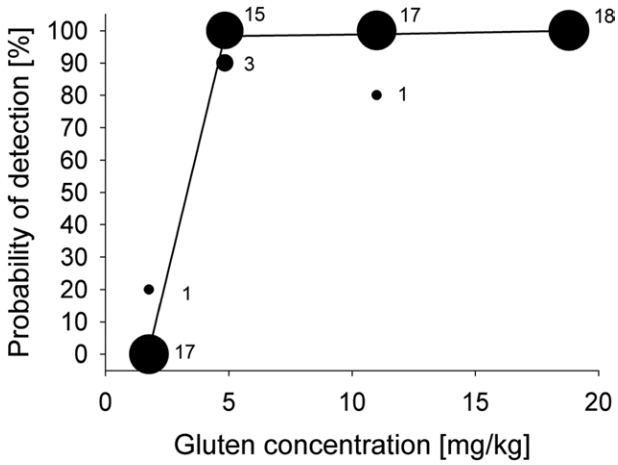

Figure 1. POD observed by each of 18 participating laboratories for samples extracted with ethanol (part A) between 1.76 and 18.8 mg/kg gluten. Number stated at each circle means number of laboratories with the same POD. Areas of circles are proportional to number of laboratories.

A graphical way to show the results for both collaborative tests appears in Figure 1 (ethanol extraction) and Figure 2 (Cocktail extraction). In these figures, the probability of detection (POD) is plotted against the concentration. Note that only 10% increments are possible for the POD in this figure. The bigger the area of the circle, the more laboratories reported this POD, as indicated by the number next to the circles.

Statistical Analysis and Discussion

Following the AOAC Appendix N for the validation of qualitative methods, some method performance characteristics were calculated and are shown in Tables 3 and 4 for both collaborative tests. Reproducibility SD was in the range between 0.00 and 0.18 after ethanol extraction and between 0.00 and 0.36 after Cocktail extraction. Repeatability SD was between 0.00 and 0.13 (ethanol extraction) and 0.00 and 0.21 (Cocktail extraction). A nonprocessed sample containing 4.8 mg/kg

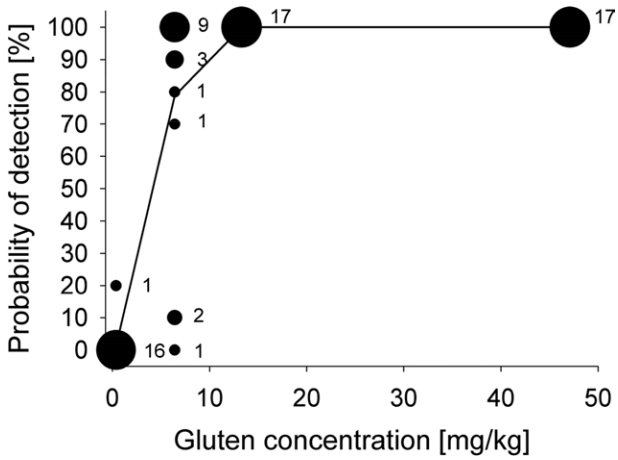

Figure 2. POD observed by each of 18 participating laboratories for samples extracted with Cocktail solution (part B) between 0.38 and 47.1 mg/kg gluten. Number stated at each circle means number of laboratories with the same POD. Areas of circles are proportional to number of laboratories.

Table 3. Performance statistics for overall results using the R5 dipstick after ethanol extraction[a]

Gluten, mg/kg	Sample 1 (negative) 1.76		Sample 2 (low) 4.84		Sample 3 (medium) 11.0		Sample 4 (high) 18.8	
	Positive	Total	Positive	Total	Positive	Total	Positive	Total
Total (18 laboratories)	2	180	177	180	178	180	180	180
POD[b]		0.01		0.98		0.99		1.00
LCL[c]		0.00		0.95		0.96		0.98
UCL[d]		0.04		0.99		1.00		1.00
s_r[e]		0.10		0.13		0.10		0.00
s_R[f]		0.11		0.18		0.11		0.00

[a] Part A (*see also* Table 1).

[b] POD = Probability of detection.

[c] LCL = Lower limit of the confidence interval.

[d] UCL = Upper limit of the confidence interval.

[e] s_r = Repeatability standard deviation.

[f] s_R = Reproducibility standard deviation.

gluten is detected with a POD of 0.98 (confidence interval from 0.95 to 0.99), whereas a processed sample with 6.4 mg/kg gluten is detected with a POD of 0.79 (confidence interval from 0.72 to 0.84). This clearly indicates the high suitability of the assay to detect contaminated samples lower than the threshold of 20 mg/kg. A more detailed statistical analysis, especially on LOD and its prediction intervals, is available elsewhere (18).

Discussion

The immunochromatographic method that was evaluated in this collaborative study was designed to detect gluten at levels clearly less than the threshold of 20 mg/kg gluten. A qualitative method to detect gluten will only result in a yes or no answer, but a user of this system needs to know with a given confidence (*1*) what minimal concentration is present if the result is positive and (*2*) what maximum amount of gluten may be present when the result is negative. From the data it can be concluded that the immunochromatographic dipstick RIDA QUICK Gliadin is capable of detecting gluten in processed and nonprocessed samples below the threshold of 20 mg/kg. A further characterization of the analytical performance of this assay, for example, LOD are given elsewhere (18). If a trained potential user works in a gluten-free laboratory and set up a quality-control plan by using control samples, the results obtained with the described method will be comparable to the results of the participating laboratories.

Table 4. Performance statistics for overall results using the R5 dipstick after Cocktail extraction[a]

Gluten, mg/kg	Sample 5 (negative) 0.38		Sample 6 (low) 6.40		Sample 7 (medium) 13.3		Sample 8 (high) 47.1	
	Positive	Total	Positive	Total	Positive	Total	Positive	Total
Total (17 laboratories)	2	170	134	170	170	170	170	170
POD[b]		0.01		0.79		1.00		1.00
LCL[c]		0.00		0.72		0.98		0.98
UCL[d]		0.04		0.84		1.00		1.00
s_r[e]		0.10		0.23		0.00		0.00
s_R[f]		0.11		0.42		0.00		0.00

[a] Part B (*see also* Table 2).

[b] POD = Probability of detection.

[c] LCL = Lower limit of the confidence interval.

[d] UCL = Upper limit of the confidence interval.

[e] s_r = Repeatability standard deviation.

[f] s_R = Reproducibility standard deviation.

Conclusions

Results from samples extracted with ethanol were uniform among laboratories, and 14 of 18 laboratories showed no false-positives or false-negatives. For Cocktail-extracted processed samples, still 9 of 17 laboratories reported no false-negative or false-positive results. In total, 4 of 350 samples were detected as false positive. A nonprocessed sample with a concentration of 4.8 mg/kg gluten was detected with an overall POD of 0.98, whereas processed samples with gluten concentrations of 6.4 and 13.3 mg/kg resulted in POD values of 0.79 and 1.0, respectively. Because the data show that the immunochromatographic dipstick RIDA QUICK Gliadin is suitable to detect gluten clearly below the CODEX threshold of 20 mg/kg, the study director, Katharina Scherf, together with the method developers from R-Biopharm, recommends this method for First Action *Official Methods of Analysis*.

Acknowledgments

We thank Carrie Maune (Trilogy Lab, Washington, MO) for preparing the samples and Nathalie Widmann and Helene Bayer (R-Biopharm AG, Darmstadt, Germany) for homogeneity testing and blinding the samples. We thank Patricia Meinhardt (R-Biopharm Inc., Washington, MO) for proofreading the manuscript and Peter Koehler (German Research Center for Food Chemistry, Freising, Germany) and Clyde Don (Foodphysica, Driel, The Netherlands) for their helpful contribution to this successful study.

We acknowledge the participation of the following laboratories in the collaborative study:

Paulo Da Costa, Nestlé Research Center, Lausanne, Switzerland

Guenther Augustin, Dr. Schär S.r.l., Postal, Italy

Sandor Tömösközi, University of Technology and Economics, Budapest, Hungary

Peter Koehler, German Research Center for Food Chemistry, Freising, Germany

Roberto Lattanzio, Eurofins, Hamburg, Germany

Tuula Sontag-Strohm, University of Helsinki, Helsinki, Finland

Joe Baumert, Food Allergy Research and Resource Program (FARRP), University of Nebraska, Lincoln, NE

Mia Hallgren, National Food Agency, Uppsala, Sweden

Lukas Kraft, R-Biopharm AG, Darmstadt, Germany

Rupert Hochegger, Agentur Gesundheit Ernährungssicherheit (AGES), Wien, Austria

Gavin O'Connor, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium

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Fernando Chirdo, Facultad de Ciencias Exactas, La Plata, Argentina

Paul Wehling, General Mills, Golden Valley, MN

Dan Thompson, Eurofins, Metairie, LA

Terry Koerner, Health Canada, Ottawa, Canada

Janette Gelroth, AIB International, Manhattan, KS

Helen Brown, Campden BRI, Chipping Campden, United Kingdom

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AOAC Official Method 2015.17
Estimation of Withanolides
(Withanoside IV, Withanoside V, Withaferin A,
12-Deoxywithastromonolide, Withanolide A, Withanolide B)
in *Withania somnifera*
Liquid Chromatography
First Action 2015

A. Principle

Withanolides are compounds specific to *Withania somnifera*. The method proposes extraction of withanolides from the sample matrix using methanol and separating the compounds using gradient high performance liquid chromatography on a C-18 column and measure in UV at 227 nm.

B. Apparatus

(**a**) *LC system*.—Shimadzu high performance liquid chromatographic system equipped with LC10A pump with SPD-M 10Avp photodiode array detector or UV detector in combination with Class-VP software or LC 2010 A and LC 2010HT integrated system equipped with quaternary gradient, autoinjector in combination with Lab Solution software or any other suitable HPLC system with similar configuration can be used.
(**b**) *Column*.—Phenomenex Luna C18(2), 250 × 4.6 mm with 5 µm particle size; Part No. 00G-4252-E0 (Phenomenex, Torrance, CA, USA; www.phenomenex.com).
(c) *Analytical balance.*—Readability, 0.1 mg.
(**d**) *Filtration apparatus*.—0.45 µm nylon filter.
(**e**) *Ultrasonic bath.*
(f) *Syringe filter.*–0.45 µm PES filter.

C. Reagents

(**a**) *Degassed mobile phase*.—(1) Dissolve 0.136 g of anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) in 900 mL HPLC grade water (obtained from Millipore, Milli-Q Water purification system) and add 0.5 mL orthophosphoric acid. Make up to 1000 mL with water, filter through 0.45 µ membrane and degas in a sonicator for 3 minutes (Solvent A).
(2) Acetonitrile (Solvent B).—

Time (min)	Solvent A Conc	Solvent B Conc
0.01	95.0	5.0
18.0	55.0	45.0
25.0	20.0	80.0
28.0	20.0	80.0
35.0	55.0	45.0
40.0	95.0	5.0
45.0	95.0	5.0

(**b**) *Diluent*.—Methanol.
(**c**) Individual *Withanolide standards.*—M/s Natural Remedies Pvt. Ltd. (Bangalore, KA, India; www.phytocompounds.com), or other suppliers.

D. Standards

Weigh accurately each 5 mg of withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A, and withanolide B reference standards to 50 mL volumetric flask. Dissolve in 10 mL methanol with the aid of gently heating and cool then make up to 50 mL with methanol.

E. Preparation of Test Solutions

(**a**) *Raw material*.—Weigh accurately a sample quantity of *Withania somnifera* raw material equivalent 5 mg (about 2.5 g will be sufficient) of withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A and withanolide B in a 250 mL beaker. Extract with 100 mL methanol boiling on water bath for 10-15 minutes and repeat the procedure 3-4 times until the raw material is completely extracted or till the extracts turn colorless. Combine all the fractions, concentrate and make up the volume to 50 mL with methanol. Filter through 0.45 microns membrane filter paper.
(**b**) *Standardized (common) extract*.—Weigh accurately a sample quantity of *Withania somnifera* extract equivalent 5 mg (about 0.5 g will be sufficient) of withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A and withanolide B in a 250 mL beaker. Extract with 100 mL methanol boiling on water bath for 10-15minutes and repeat the procedure 3-4 times until the raw material is completely extracted or the extracts turn colorless. Combine all the fractions, concentrate and make up the volume to 50 mL with methanol. Filter through 0.45 microns membrane filter paper.

F. Analysis

(**a**) *Chromatographic conditions*.—*Column*.—Phenomenex Luna C18(2), 250 × 4.6 mm with 5 µm particle size; Part No. 00G-4252-E0. *Temperature*: Maintained at a constant temperature between 20 to 30°C (preferably 27°C). *Detector*: SPD-M 10Avp photodiode array detector or UV detector. *Wavelength*: 227 nm. *Flow rate*: 1.5 mL/min. *Run time*: 45 minutes.

(**b**) *Retention time relative*.—Relative retention time of Withanoside IV, 0.7; withanoside V, 0.89; withaferin A, 0.92; 12-deoxywithastramonolide, 0.96; withanolide A, 1.0; withanolide B, 1.15.

(**c**) *System suitability*.—(*1*) *Repeatability.*—The RSD of each of the individual withanolides peak area for at least 5 consecutive injections of the level 4 linearity standard solution must be ≤2.5%.

(*2*) *Resolution*.—Calculate the resolution between withanoside V andwWithaferin A peaks as follows:

$$R = 2 \times \frac{T2 - T1}{W1 + W2}$$

where T1 and T2 are the retention times of withanoside V and withaferin A respectively and W1 and W2 are their peak widths measured at the baseline between tangents drawn to the peak sides. The resolution between ephedrine and pseudoephedrine in should be ≥3.0.

(*3*) *Tailing.*—Calculate the tailing factor (F) as follows:

$$F = \frac{L + R}{2L}$$

where L is the width from start of the peak to the perpendicular from the peak apex at 5% of the peak height; R is the width from the perpendicular from the peak apex to the peak end at 5% of the peak height. The tailing factor must be ≤1.5 for all individual withanolides in the linearity standard solution chromatograms.

(*4*) *Determination coefficient*.—The r^2 for the regression line of peak area vs. concentration for each withanolide must be ≥0.998.

Procedure: Inject three times the standard preparation and calculate the mean area and the RSD. The RSD should not be more than 2%. Inject 20 µL of sample preparation and record the chromatogram at 227 nm. Calculate the percentage of withanoside IV, withanoside V, withaferin A, 12-deoxywithastramonolide, withanolide A and withanolide B content from the peak areas using the formula:

Peak integration: Base to base

Individual withanolide (%w/w)

$$= \frac{\text{Area of the sample}}{\text{Area of the standard}} \times \frac{\text{Weight of standard (mg)}}{\text{Standard dilution}} \times \frac{\text{Sample dilution}}{\text{Sample weight (mg)}} \times \text{Purity of standard \%}$$

Compound name	CAS No.
Withanoside IV	362472-81-9
Withanoside V	256520-90-8
Withaferin A	5119-48-2
12-Deoxywithastramonolide	60124-17-6
Withanolide A	32911-62-9
Withanolide B	56973-41-2

References: AOAC SMPR 2015.007
J. AOAC Int. **98**, 1104(2015)
DOI: 10.5740/jaoac.int.SMPR2015.007

J. AOAC Int. (future issue)

Posted: January 19, 2016

AOAC Official Method 2015.18

Phosphorus and Potassium in Commercial Inorganic Fertilizers

Inductively Coupled Plasma-Optical Emission Spectrometry

First Action 2015

[Applicable for the determination of both "citrate-EDTA soluble" phosphorus and potassium (Alternative A) and "acid-soluble" phosphorus and potassium (Alternative B) in commercial inorganic fertilizers by ICP-OES. Citrate-EDTA soluble phosphorus and potassium (Alternative A) is directly synonymous with "available phosphate" and "soluble potash" respectively. Acid-soluble is sometimes referred to as "total" phosphorus and potassium; however, Alternative B may under-estimate the total phosphorus and potassium content if acid insoluble compounds are present. Repeatability for citrate soluble P and K, expressed as % RSD, ranges from 0.28 to 1.30 for P and from 0.41 to 1.52% for K. Repeatability for acid soluble P and K, expressed as % RSD, ranges from 0.71 to 1.13 for P and from 0.39 to 1.18% for K. *For liquid fertilizers containing phosphite and for organic fertilizers, an alternative AOAC method such as **960.03** or **993.31** should be used as the ICP-OES will recover phosphorus that is not considered readily plant available in these materials.*]

Alternative A: Neutral Ammonium Citrate–Disodium EDTA-Soluble P and K Using ICP-OES

A. Apparatus

(a) *Analytical balance*.—Readability to 0.1 mg, AT 200 (Mettler Toledo, Columbus, OH), or equivalent.

(b) *pH meter*.—Readability to pH 7.00, Model 8005 (VWR Scientific, Radnor, PA), or equivalent.

(c) *pH combination electrode*.—Orion 9102BNWP (Thermo Fisher Scientific, Waltham, MA), or equivalent.

(d) *Constant temperature water bath*.—Capable of maintaining bath temperatures of 65 ± 2°C, BK53 (Yamato Scientific, Santa Clara, CA), or equivalent.

(e) *Heated shaking water bath*.—Capable of maintaining bath temperatures of 65 ± 2°C, and set to approximately 200 reciprocations/min.

(f) *ICP-OES instrument*.—Vista Pro-axial view (Agilent Technologies, Santa Clara, CA), or equivalent.

(g) *Gated riffle splitter*.—Model 16-25X (Carpco Inc., Jacksonville, FL), or equivalent.

(h) *Grinding mill*.—Model ZM200 rotor mill (Retsch, Haan, Germany), or equivalent.

B. Reagents

(a) *Ammonium citrate, dibasic*.—$(NH_4)_2HC_6H_5O_7$, formula weight (FW) 226.19, ACS grade, purity >98% (EMD Chemicals, Darmstadt, Germany).

(b) *EDTA, disodium salt, dihydrate*.—$C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$, FW 372.24, purity > 99% (J.T. Baker Chemicals, Center Valley, PA).

(c) *Ammonium hydroxide*.—NH_4OH, FW 35.05, 28.0–30.0% as NH_3 (Mallinckrodt Chemicals, Center Valley, PA).

(d) *Nitric acid*.—HNO_3, 67–70%, OmniTrace grade (EMD Chemicals).

(e) *Potassium dihydrogen phosphate*.—KH_2PO_4, certified at 22.73% P and 28.73% K, National Institute of Standards and Technology (NIST) 200a (Gaithersburg, MD), www.nist.gov/srm.

(f) *Potassium chloride*.—KCl, FW 74.55, ACS grade, purity >99% (Mallinckrodt Chemicals).

(g) *Potassium nitrate*.—KNO_3, certified at 38.66% K, NIST 193.

(h) *Triton X-100*.—Octylphenol ethoxylate (J.T. Baker Chemicals).

(i) *10, 000 µg/mL Be standard*.—In 4% HNO_3, product 10M5-1 (High Purity Standards, Charleston, SC).

(j) *10,000 µg/mL Sc standard*.—In 4% HNO_3, product 10M48-1 (High Purity Standards).

(k) *Cesium chloride*.—CsCl, FW 168.36, purity > 99.999% (Sigma-Aldrich, St. Louis, MO).

(l) *Lithium nitrate*.—$LiNO_3$, FW 68.95, purity > 99%, (EM Science, Gibbstown, NJ).

(m) *Citrate–EDTA extraction solution (0.11 M ammonium citrate and 0.033 M disodium EDTA)*.—Weigh and completely transfer 25 g disodium EDTA [(**b**) above] and 50 g dibasic ammonium citrate [(**a**) above] to a 2 L volumetric flask containing approximately 1500 mL deionized (or equivalent) water. Adjust the pH to near neutral by adding 30 mL of (1 + 1, v/v) ammonium hydroxide [(**c**) above] –water solution in a fume hood. Adjust the final pH to 7.00 (±0.02) using a pH electrode [Alternative A, A (**b**)] and meter [Alternative A, A (**c**)] while adding the ammonium hydroxide–water (1 + 1, v/v) solution drop-by-drop and stirring. After obtaining a stable pH of 7.00 (±0.02), bring the flask to volume with deionized water and mix. Larger volumes of this solution can be prepared; however, it is susceptible to microbial degradation resulting in a maximum shelf life of 2 weeks, if stored in a dark location.

(n) *0.5% Triton-X*.—Add 1 mL Triton X-100 [(**h**) above] to a 200 mL volumetric flask and dilute to volume with deionized water.

(o) *Internal standard/ionization buffer (10 µg/mL Sc in 0.018 M CsCl and 4% nitric acid)*.—

Add 1 mL 10 000 µg/mL Sc stock standard [(**j**) above], 3 g cesium chloride [(**k**) above], 20 mL nitric acid [(**d**) above], and 1 mL 0.5% Triton-X [(**n**) above] to a 1 L volumetric flask containing approx. 500 ml deionized (or equivalent) water. Bring flask to volume with deionized (or equivalent) water and mix. If Be is used as an internal standard, add 4 mL of 10 000 µg/mL Be [(**i**) above] stock standard to obtain a concentration of 40 µg/mL Be.

(p) *2500 µg/mL Phosphorus as orthophosphate (PO_4).*–Commercial custom standard prepared in a water matrix preserved with a biocide (Inorganic Ventures, Christiansburg, VA, USA). Note a commercial stock standard preserved in acid is not acceptable as the acid will change the matrix of the pH neutral ammonium citrate EDTA and produce erroneous results.

(q) *7500 µg/mL Potassium from potassium chloride.*–Commercial custom standard prepared in a water matrix preserved with a biocide (Inorganic Ventures). Note a commercial stock standard preserved in acid is not acceptable as the acid will change the matrix of the pH neutral ammonium citrate EDTA and produce erroneous results.

C. Calibration

(a) *Standard solution*.—Prepare calibration standards from potassium dihydrogen phosphate [Alternative A, B(**e**)], potassium chloride [Alternative A, B(**f**)], and potassium nitrate [Alternative A, B(**g**)] as recommended in Table **2015.18A**. Several calibration standards are required since multiple ICP-OES wavelengths are utilized, since some wavelengths are split into multiple calibration segments, and since a minimum of five points per curve is recommended. Table **2015.18A** provides the P and K concentrations, expressed as µg/mL and the percent oxide forms.

(b) *Stock standards*.—A 2500 mg/L custom blend commercial phosphorus standard [Alternative A, B(**p**)] and a 7500 custom blend commercial potassium standard [Alternative A, B(**q**)] can also be used, but commercial stock standards preserved in acid should not be used as the acid changes the pH and matrix of the calibration standards and can produce erroneous results. Table **2015.18B** provides the details for preparing standards from custom purchased standards.

(c) *ICP-OES calibration*.—Emission intensity for each of the calibration standards is plotted against concentration. A minimum of five calibration standards is recommended for each wavelength. Use an internal standard [Alternative A, B(**o**)] to adjust the concentration of the calibration standards and test solutions. The recommended wavelengths, standards, concentration ranges, curve fit, and neighboring wavelengths that may produce spectral interference are listed in Table **2015.18C**. Linear regression is preferred, whenever possible. Quadratic curve fit may be necessary because of the dynamic range in fertilizer K concentration,

but ensure that the curvature is not excessive. Many ICP software programs have algorithms to detect excessive curvature of second order or quadratic calibration curves. Alternatively, linear calibration can be achieved by removal of the high concentration K standards; however, secondary dilution of high concentration test solutions will be required. Dilutions must maintain the solvent matrix, which is prepared by diluting 400 mL citrate–EDTA extract solution [Alternative A, B (**m**)] to 1 L.

(d) *Empirical calibration (optional).*–The combination of an organic solvent, high salts and high Phosphorus in the test portion can result in suppression of signal intensity. This method is designed to address these issues by matrix and aliquot dilution using the recommended pump tube configuration, plus the use of robust plasma conditions and an internal standard. However, if this recommended configuration still produces low phosphorus recoveries for the fertilizer concentrates (i.e. 40-52% P_2O_5), then empirical calibration may be necessary. Fertilizer concentrates with certified or accepted consensus values can be obtained from Laboratory Quality Services International (LQSI) (www.sgs.com/en/mining/Analytical-Services/Proficiency-Testing-Programs-LQSi.aspx), and the Magruder (www.magruderchecksample.org) or AFPC (www.afpc.net) check sample programs. Note that calibration solutions obtained from these certified or consensus reference materials are prepared by following the recommended extraction procedure (Alternative A, E) and that these standards can only be used for calibration within the batch of test solutions with which they were extracted. These standard extract solutions have the same shelf life (i.e. approx. 16 h) as the other fertilizer extracts and must be prepared fresh with each run. Calculations for converting the % P_2O_5 in these materials to mg/L P are provided in the Calculations section (Alternative A, G). Fertilizer materials below 40% P_2O_5 (or approx. 350 µg/mL P) typically do not experience this suppression issue, so standards below this concentration can be obtained using those listed in Tables **2015.18A** or **B**. Empirical calibration is not the preferred option and should only be used as a last resort.

The test solution and internal standard/ionic buffer solution [Alternative A, B (**o**)] are blended using a Y-connector (Part 30703-90; Cole-Parmer, Bunker, CT) just prior to the nebulizer using the conditions described in Table **2015.18D**.

D. Sample Preparation

Collect a primary field sample using one of the recommended AOAC sampling procedures (i.e. **929.01, 969.01**, or **992.33**) or other recognized protocol. Prepare solid fertilizer materials by riffling [Alternative A, A (**g**)] the entire 5 lb (2300 g) laboratory sample twice before further

riffling down to collect an approximate 100 g subsample. The entire 100 g subsample is ground [Alternative A, A (**h**)] to pass a 0.75 mm mesh screen. The ground analytical sample is placed into a 1 qt (0.946 L) glass jar and mixed by careful rotation and inversion. Use a coring device or spoon to transfer sufficient mass to fill a 3 oz (90 mL) glass jar approximately half full. For liquid materials, shake the laboratory sample vigorously to ensure thorough mixing, and then pour sufficient volume into a 3 oz. (90 mL) glass jar to fill it approximately half full. Test portions for analyses are taken from the 3 oz (90 mL) jar after inverting and rotating the jar to ensure contents are thoroughly mixed. Other validated sample preparation techniques that result in a representative and homogeneous test portion are also acceptable.

E. Extraction

Weigh a 0.5 ± 0.01 g prepared fertilizer test portion (Alternative A, D) and completely transferred to a 250 mL wide-mouth class A volumetric flask. Dispense 100 mL of 65 ± 2°C preheated citrate– EDTA extraction solution [Alternative A, B (**m**)] into each flask and insert a rubber stopper. Shake test solutions in a 65 ± 2°C preheated water bath set to approximately 200 reciprocations per minute for exactly 60 min; then remove from the water bath, allow to cool to room temperature (20 to 25°C), bring to volume with deionized (or equivalent) water, stopper, and mix. Filter any test solution containing suspended debris using P and K free filters. Due to a very limited shelf life, analyze test solutions within 16 h of extraction.

F. ICP Conditions

The optimal instrument conditions identified during method validation of citrate–EDTA-soluble P and K are listed in Table **2015.18D**. Monitor rinse time and buffer concentration closely as they are sensitive to change (1).

ICP-OES differ in their design and options, so minor adjustment to the conditions listed in Table **2015.18D** may be necessary; however, any adjustments to these conditions must be performance based and validated. Special attention should be paid to the recovery of phosphorus in fertilizer concentrates such as MESZ^{TM} (40% P_2O_5), DAP (46% P_2O_5) and MAP (50% or 52% P_2O_5) since these materials pose the greatest need for optimal instrument performance.

G. Calculations (Alternative A)

Several variables exist in the instrument software for data reporting, including units, test portion weight, test solution volume, and dilution factor. The calibration standards are prepared as µg/mL of P and K, and the final fertilizer results are reported as percentage P_2O_5 and K_2O, which requires the following calculation:

$$P_2O_5, \% = [P \times (250/W) \times 142/(31 \times 2)]/10000$$

where P = ICP-OES P reading in µg/mL, 250 = final volume in mL, W = test portion weight in g, 142 = FW of P_2O_5, 31 = FW of P, 2 = mole ratio of P_2O_5/P, and 10 000 = conversion of % to µg/mL.

$$K_2O, \% = [K \times (250/W) \times 94.2/(39.1 \times 2)]/10000$$

where K = ICP-OES K reading in µg/mL, 250 = final volume in mL, W = test portion weight in g, 94.2 = FW of K_2O, 39.1 = FW of K, 2 = mole ratio of K_2O/K, and 10 000 = conversion of % to µg/mL.

Alternatively, the standards can be entered with their theoretical percentage P_2O_5 and K_2O in solution values, listed in Tables **2015.18A** and **B**.

If empirical calibration [Alterative A, C (**d**)] is used, conversion of the %P_2O_5 in the certified or consensus material to mg/L P in the calibration solution is obtained using the following equation:

$$P, \mu g/mL = \% P_2O_5 \times 10{,}000 \times (W/250) \times [(31 \times 2)/142]$$

where P, µg/ mL = P concentration in the extracted standard solution; % P_2O_5 = the certified or consensus value, 10 000 = conversion of % to µg/mL, W = test portion weight in g, 250 = final volume in mL, 31 = FW of P, 2 = mole ratio of P_2O_5/P, and 142 = FW of P_2O_5.

H. Comments (Alternative A)

Relative to other AOAC methods (**960.03, 978.01 and 993.01**), the ICP-OES can produce lower phosphorus recoveries and/or greater data variability (www.magrudercheckssample.org). A list of critical factors and common error sources are listed here. For phosphorus, three issues are critical – addressing matrix challenges, implementing robust plasma conditions, and utilizing

proper standards. Carbon in the citrate and EDTA will reduce the plasma efficiency, so it must be addressed. Diluting the matrix by using a smaller sample pump tube and a larger internal standard/ionization buffer pump tube as listed in Table **2015.18D** is the approach used in this method. Other options include: 1) the use of oxygen addition to the argon to help combust the carbon, 2) a separate manual dilution of the test solutions and standards in a 4% nitric acid acid solution, or 3) a complete destruction of the carbon with a secondary digestion of the extract solution in nitric acid. Other factors that can help improve Phosphorus recoveries include configurations that decrease the volume of aerosol injected into the plasma, such as a slower pump speed, slightly lower nebulizer pressure, and/or a double path or baffled spray chamber. Finally, the final matrix of the calibration standards and test solutions much match closely. Standards prepared from salts as provided in Table **2015.18A** have the closest match and offer the best Phosphorus recoveries. If commercial stock standards are used, a source of P as PO_4^{x-} that is in a matrix that will not adversely change the pH neutral ammonium citrate-EDTA matrix works best. Stock standards preserved in acid solution are not recommended.

Potassium is easily ionized and generally poses fewer problems than phosphorus. The greatest challenge with potassium is capturing the broad concentration range found in fertilizers, since it produces an intense signal resulting in a limited linear dynamic range. If possible, potassium should be read in the radial mode, and it may benefit from slightly lower nebulizer pressures and pump speeds. As described in Table **2015.18C**, the use of multiple wavelengths (766, 769 and 404 nm) and/or multiple calibration segments to cover the dynamic concentration range is recommended. Quadratic curve fit can help expand the useful range of some of these wavelengths, but great caution should be exercised to ensure the curve falls within the sensitive response range without excessive curvature. Also, secondary dilution of high concentration test solutions can help.

Deviation from this method is not recommended, but if small revisions are necessary to accommodate differences in ICP-OES types and design, then these revisions should be validated.

Alternative B: Acid Soluble P and K Using ICP-OES

A. Apparatus (Alternative B)

(a) *Balance*.—Readability to 0.1 mg, Sartorius BP210S (Gottingen, Germany), or equivalent.

(b) *Hot plate*.—Model 53015, Lindburg/Blue M (Watertown, WI), or equivalent.

(c) *ICP-OES instrument*.—Thermo 6500 Duo View (Thermo Scientific, Cambridge, UK), or equivalent.

(d) *Gated riffle splitter*.—SP-177 Jones Standard Aluminum Splitter (Gilson Co., Inc., Lewis Center, OH), or equivalent.

(e) *Grinding mill*.—Mikro-Samplemill (Pulverizing Machinery, Summit, NJ), or equivalent.

B. Reagents (Alternative B)

(a) *Hydrochloric acid*.—HCl, 35–38%, trace metal grade, Cat. No. A508-500 (Fisher Scientific, Pittsburgh, PA).

(b) *Ammonium dihydrogen phosphate*.—$NH_4H_2PO_4$, FW 115.03, trace metal basis, purity >99.999%, Cat. No. 204005-100G (Sigma-Aldrich).

(c) *Potassium chloride*.—KCl, FW 74.55, trace metal basis, purity > 99.99%, Cat. No. 204099-250G (Sigma-Aldrich).

(d) *Scandium oxide*.—Sc_2O_3, FW 137.91, Item No. OX21-5N (Stanford Materials Corp., Irvine, CA).

(e) *Nitric acid*.—HNO_3, 69.2%, certified ACS plus grade, Cat. No. A200 C212 (Fisher Scientific).

(f) *Triton X-100*.—Polyethylene-glycol *p*-tert-octylphenyl ether, 4-$(C_8H_{17})C_6H_4(OCH_2CH_2)_nOH$ (*n* approximately 10), FW 624, Cat. No. BP151-500 (Fisher Scientific).

(g) *Cesium chloride*.—CsCl, FW 168.36, trace metal basis, purity >99.999%, Cat. No. 203025-50G (Sigma-Aldrich).

(h) *Lithium nitrate*.—$LiNO_3$ ReagentPlus grade, FW 68.95, Cat. No. 227986-1KG (Sigma-Aldrich).

(i) *10000 µg/mL Be stock standard*.—In 5% HNO_3, Cat. No. PLBE-10-500 (Exaxol Corp., Clearwater, FL).

(j) *10000 µg/mL Sc stock standard*.—Weigh 15.3374 g scandium oxide [(**d**) above] into a 600 mL beaker. Add 300 mL deionized water, then slowly add 100 mL nitric acid [(**e**) above]. Heat solution on a hotplate to a gentle boil, and continue boiling until the solution becomes clear.

(k) *1% Triton X*.—Pipet 10 mL Triton X-100 solution [(**f**) above] into a 1 L flask. Bring to volume with deionized (or equivalent) water and mix.

(l) *Internal standard/ionization buffer (60 µg/mL Sc in 0.035 M CsCl and 2% HNO_3)*.—Add 6 mL 10000 µg Sc/mL stock standard [(**j**) above], 6 g CsCl [(**g**) above], 20 mL HNO_3 [(**e**) above], and 2 mL 1% Triton X [(**k**) above] to a 1 L flask containing approximately 500 mL deionized (or equivalent) water. Bring to volume with deionized water and mix. If $LiNO_3$ is used as the ionic buffer, replace the CsCl with 8 g $LiNO_3$ [(**h**) above]. If Be is used as an internal standard, add 1

mL 10 000 µg/mL Be stock standard solution [(**i**) above] to obtain a 10 µg/mL Be internal standard concentration.

(m) *4 M Hydrochloric acid digestion solution*.—Add approx. 500 mL deionized (or equivalent) water to a water to a 1 L volumetric flask. Slowly add 333 mL of concentrated hydrochloric acid [(**a**) above], then bring to volume with deionized water and mix.

C. Calibration (Alternative B)

(a) *Standard solution.*—Prepare calibration standards from ammonium dihydrogen phosphate [Alternative B, B(**b**)] and potassium chloride [Alternative B, B(**c**)] as recommended in Table **2015.18E**. As with Alternative A, many calibration standards are required since multiple ICP-OES wavelengths are used, since some wavelengths are split into multiple calibration segments, and since a minimum of five points/curve is recommended. Table **2015.18E** provides the P and K concentrations expressed as µg/mL and their percent oxide forms.
Better P recoveries were obtained using weighed salts [Alternative B, B(**b**)], so commercially available stock standard solutions are not recommended.

(b) *ICP-OES calibration.*—Emission intensity for each of the calibration standards is plotted against concentration. A minimum of five calibration standards are used for each wavelength. Use an internal standard [Alternative B, B(**l**)] to adjust the concentration of the calibration standards and test solutions. The wavelengths, standards used, concentration ranges, curve fit, and wavelengths that may require spectral deconvolution are listed in Table **2015.18F**. The data in Table **2015.18F** are based on a radial view for K. If linear regression to 1000 µg/mL of K is not possible, one or more of the following will be necessary: selecting quadratic curve fit (provided the curvature is not excessive), utilizing wavelength 404.721 nm for the five highest K calibration standards listed in Table **2015.18E**, dropping one or more of the top K standards listed in Table **2015.18E** and/or conducting dilutions of the test solutions using 0.16 M HCl.

The test solution and internal standard/ionic buffer solutions are blended using a reagent “T” connector (Part No. 116-0522-01, Bran+Luebbe, Mequon, WI) just prior to the nebulizer using the conditions described in Table **2015.18G**.

D. Sample Preparation

Collect a primary field sample using one of the recommended AOAC sampling procedures (**929.01**, **969.01**, or **992.33**) or other recognized protocol. Prepare solid materials by riffling [Alternative B, A(**d**)] the entire 5 lb (2300 g) laboratory sample three times before further riffling down to an approximate 100 g subsample. The entire 100 g subsample is then ground [Alternative B, A(**e**)] to pass a Tyler-35 mesh sieve (U.S. Standard Sieve Size No. 40, 0.420 mm or 0.165 in. opening; Fisherbrand stainless steel; Fisher Scientific, Pittsburgh, PA). The ground analytical sample is placed into a 1 quart (0.946 L) glass jar and mixed by careful rotation and inversion. Use a coring device or spoon to transfer sufficient mass to fill a 3 ounce (90 mL) glass jar approximately half full. For liquid materials, shake the laboratory sample vigorously to ensure thorough mixing, and then pour sufficient volume into a 3 oz (90 mL) glass jar to fill it approximately half full. Test portions for analyses are taken from the 3 oz (90 mL) jar after inverting and rotating the jar to ensure contents are thoroughly mixed. Other validated sample preparation techniques that result in a representative and homogeneous test portion are also acceptable.

E. Extraction (Alternative B)

Weigh a 0.5 ± 0.01 g fertilizer test portion and completely transfer to a 250 mL class A volumetric flask. Slowly add 30 mL of deionized (or equivalent) water to each flask. Dispense 10 mL o f 4 M HCl digestion solution [Alternative B, B(**m**)] into the flask. Place flasks on a preheated hotplate and gently boil for 15 ± 1 min. Remove individual flasks that have boiled for 15 ± 1min and allow them to cool to room temperature (20 to 25°C). Bring flasks to volume with deionized (or equivalent) water. Filter any test solution containing suspended debris using P and K free filters. The final acid strength of the test solution is approximately 0.16 M HCl, so any test solutions requiring dilution should be prepared in 0.16 M HCl. Due to a limited shelf life, all analyses should occur within 2 weeks of digestion. After repeated heating and cooling cycles of the 250 mL volumetric flasks, check the calibration of the flasks by adding 250 g of deionized (or equivalent) water and verify that the volume is at the meniscus. When a flask loses calibration, either use the corrected volume established by water weight, or discard it.

F. ICP-OES Conditions

Limit the deviation from a test portion weight of 0.5 g to ±0.025 g. Also, K is sensitive to nebulizer pressure/flow, so closely monitor the nebulizer condition, as they can deteriorate over

time. Instrument conditions used for method validation of acid-soluble/total P and K are listed in Table **2015.18G**. ICP-OES differ in their design and options, so minor adjustment to the conditions listed in Table **2015.18G** may be necessary; however, any adjustments to these conditions should be performance based and validated. Special attention should be paid to the recovery of phosphorus in fertilizer concentrates such as MESZ^{TM} (40% P_2O_5), DAP (46% P_2O_5) and MAP (50% or 52% P_2O_5) since these materials pose the greatest need for optimal instrument performance.

G. Calculations Alternative B

See Calculations for Alternative A.

H. Comments (Alternative B)

The 0.16 M HCl matrix used in Alternative B poses fewer analytical challenges for the ICP-OES than does the citrate-EDTA solvent used in Alternative A. If minor method modifications are necessary to accommodate different ICP-OES types or designs and/or to correct for variable or low phosphorus recoveries, the following are likely watch areas. Increasing the plasma power often benefits phosphorus. Decreasing the volume of the aliquot injected into the plasma can also help improve recoveries of materials containing high concentrations of phosphorus. This can be accomplished by using a smaller sample pump tube and/or larger internal standard/ionization buffer pump tube, and/or by slightly decreasing the pump speed and/or nebulizer pressure. The final matrix of the test solutions and standards should closely match. Standards prepared from salts as provided in Table **2015.18E** provide the greatest match andoffer the best Phosphorus recoveries. Stock standards preserved in acid solution are not recommended. The comments provided for potassium in Alternative A section H also apply to potassium in Alternative B.

Deviation from this method is not recommended, but if small revisions are necessary to accommodate differences in ICP-OES types and design, then these revisions should be validated.

References

1. *J. AOAC Int*. **97**, 687(2014)

DOI: http://dx.doi.org/10.5740/jaoacint.12-399

J. AOAC Int. (future issue)

Posted: February 17, 2016

Table 2015.18A. ICP calibration standards from stock reagent salts for citrate–EDTA-soluble P and K

Standard ID	Volume, mL	Citrate, mL	Stock 1, mL[a]	Stock 2, mL[b]	P concn, µg/mL	P_2O_5, µg/mL	P_2O_5 solution, %	P_2O_5 fertilizer, %	K concn, µg/mL	K_2O, µg/mL	K_2O solution, %	K_2O fertilizer, %
Blank	1000	400	0	0	0	0	0	0	0	0	0	0
1	250	100	10 of Std 7	NA	12	27.5	0.00275	1.4	15.15	18.25	0.00182	0.9
2	250	100	20 of Std 7	NA	24	55	0.00550	2.7	30.3	36.5	0.00365	1.8
3	250	100	5	NA	50	115	0.01146	5.7	63.1	76	0.00760	3.8
4	250	100	10	NA	100	229	0.02291	11.5	126	152	0.01521	7.6
5	250	100	15	NA	150	344	0.03437	17.2	189	228	0.02281	11.4
6	250	100	22[c]	NA	220	504	0.05041	25.2	278	335	0.03345	16.7
7	250	100	30	NA	300	687	0.06874	34.4	379	456	0.04562	22.8
8	250	100	40	NA	400	917	0.09165	45.8	505	608	0.06083	30.4
9	250	100	50	NA	500	1146	0.11457	57.3	631	760	0.07603	38
10	250	100	NA	25	NA	NA	NA	NA	747	900	0.08998	45
11	250	100	NA	30	NA	NA	NA	NA	897	1081	0.10805	54
12	250	100	NA	35	NA	NA	NA	NA	1046	1260	0.12600	63

[a] Stock 1 = 2500 µg/mL P stock standard = 2.7461 g potassium dihydrogen phosphate (KH_2PO_4)/250 mL prepared in deionized water.

Also, 10 of Std 7 represents a serial dilution from another standard, meaning take 10 mL from standard 7.

[b] Stock 2 = 7472 µg/mL K stock standard = 3.5615 g potassium chloride or 4.8299 g potassium nitrate/250 mL in deionized water.

[c] 22 mL can be achieved by using a 15 mL and a 7 mL class A pipet or equivalent combination.

Table 2015.18B. ICP calibration standards from commercial custom blend stock standard solutions

Standard ID	Volume, mL	Citrate, mL	Stock 2500 P, mL[a]	Stock 7500 K, mL[b]	P concn, µg/mL	P_2O_5, µg/mL	P_2O_5 solution, %	P_2O_5 fertilizer, %	K concn, µg/mL	K_2O, µg/mL	K_2O solution, %	K_2O fertilizer, %
Blank	1000	400	0	0	0	0	0	0	0	0	0	0
1	250	100	10 of Std 7	NA	12	27.5	0.00275	1.4	360	434	0.04336	21.7
2	250	100	20 of Std 7	NA	24	55	0.00550	2.7	300	361	0.03614	18.1
3	250	100	5	NA	50	115	0.01146	5.7	240	289	0.02891	14.5
4	250	100	10	NA	100	229	0.02291	11.5	180	217	0.02168	10.8
5	250	100	15	NA	150	344	0.03437	17.2	120	144	0.01441	7.2
6	250	100	22[c]	NA	220	504	0.05041	25.2	63	76	0.00759	3.8
7	250	100	30	NA	300	687	0.06874	34.4	NA	NA	NA	NA
8	250	100	40	NA	400	917	0.09165	45.8	33.6	40	0.00405	2.0
9	250	100	50	NA	500	1146	0.11457	57.3	16.8	20	0.00202	1.0
10	250	100	NA	25	NA	NA	NA	NA	450	542	0.05421	27.1
11	250	100	NA	30	NA	NA	NA	NA	600	723	0.07227	36.1
12	250	100	NA	35	NA	NA	NA	NA	750	903	0.09034	45.2
13	250	100	NA	35	NA	NA	NA	NA	900	1084	0.10841	54.2
14	250	100	NA	35	NA	NA	NA	NA	1050	1265	0.12648	63.2

[a] Stock 1 = 2500 µg/mL P as PO_4 custom stock standard [see Alternative A, B(**p**)] Also, 10 of Std 7 represents a serial dilution from another standard, meaning take 10 mL from standard 7.

[b] Stock 2 = 7500 µg/mL K from KCl custom stock standard [see Alternative A, B(**q**)].

[c] 22 mL can be achieved by using a 15 mL and a 7 mL class A pipet or equivalent combination.

Table 2015.18C. Calibration criteria for "direct-available" P and "soluble" K by ICP-OES

Element ID	Wavelength, nm[a]	Calibration range, µg/mL	Standards used (*see* Table **2015.18A**)	Curve fit	Spectral deconvolution
P	177.434 (1)	0 to 100	Blank, 1, 2, 3, 4	Linear	None
P	177.434 (2)	100 to 500	4, 5, 6, 7, 8, 9	Linear	None
P	178.222 (1)	0 to 100	Blank, 1, 2, 3, 4	Linear	None
P	178.222 (2)	100 to 500	4, 5, 6, 7, 8, 9	Linear	None
P	213.618 (1)	0 to 100	Blank, 1, 2, 3, 4	Linear	Cu 213.598
P	213.618 (2)	100 to 500	4, 5, 6, 7, 8, 9	Linear	Cu 213.598
P	214.914 (1)	0 to 100	Blank, 1, 2, 3, 4	Linear	Cu 214.898
P	214.914 (2)	100 to 500	4, 5, 6, 7, 8, 9	Linear	Cu 214.898
K	766.485 (1)	0 to 126	Blank, 1, 2, 3, 4	Quadratic	None
K	766.485 (2)	50 to 379	3, 4, 5, 6, 7	Quadratic	None
K	769.897	126 to 505	4, 5, 6, 7, 8	Quadratic	Interference $LiNO_3$
K	404.721	505 to 1046	8, 9, 10, 11, 12	Quadratic	None

[a] (1) and (2) distinguish between the same wavelength used to cover two separate concentration ranges.

Table 2015.18D. Final ICP-OES conditions utilized for citrate-EDTA-soluble P and K validation

Factor	Setting
Power, kW	1.45
Plasma flow, L/min	19.5
Auxiliary flow, L/min	2.25
Nebulizer pressure, L/min	0.7
Nebulizer type	Seaspray
Spray chamber	Cyclonic
Sample pump tube	Black/black
Buffer/internal standard pump tube	Gray/gray
CsCl ionic buffer concn, M	0.018
Internal standard and concn, µg/mL	10
Buffer matrix	4% nitric acid
Exposure length, s	10
Number of exposures	3
Rinse time, s	35
Total analysis time, min	2

Table 2015.18E. ICP-OES calibration standards from stock reagent salts for "total" P and K

Standard ID	Vol., mL	Acid, mL[a]	Weight $NH_4H_2PO_4$, g	Weight KCl, g	P concn, µg/mL	P_2O_5, µg/mL	P_2O_5, solution, %	P_2O_5, sample, %	K concn, µg/mL	K_2O, µg/mL	K_2O solution, %	K_2O sample, %
Blank	1000	40	0	0	0	0	0	0	0	0	0	0
1	1000	40	40 of Standard 6[b]	0.6305	9.8	22.4	0.00224	1	332	400	0.0400	20
2	1000	36	100 of Standard 10[b]	0.4748	47	108	0.01076	5	249	300	0.0300	15
3	500	12	100 of Standard 10[b]	100 of Standard 14[b]	94	215	0.02153	11	163	196	0.0196	10
4	1000	32	0.4539	200 of Standard 12[b]	122	280	0.02802	14	116	140	0.0140	7
5	1000	36	0.6810	100 of Standard 14[b]	184	420	0.04204	21	81	98	0.0098	5
6	1000	40	0.9079	50 of Standard 13[b]	245	561	0.05605	28	34.9	42	0.0042	2
7	1000	40	1.1349	25 of Standard 13[b]	306	701	0.07007	35	17.4	21	0.0021	1
8	1000	40	1.3619	NA[c]	367	841	0.08408	42	NA	NA	NA	NA
9	1000	40	1.5888	NA	428	981	0.09809	49	NA	NA	NA	NA
10	1000	40	1.7510	NA	472	1081	0.10811	54	NA	NA	NA	NA
11	1000	40	NA	0.7915	NA	NA	NA	NA	415	500	0.0500	25
12	1000	40	NA	1.1079	NA	NA	NA	NA	581	700	0.0700	35
13	1000	40	NA	1.3295	NA	NA	NA	NA	697	840	0.0840	42
14	1000	40	NA	1.5511	NA	NA	NA	NA	814	980	0.0980	49
15	1000	40	NA	1.7727	NA	NA	NA	NA	930	1120	0.1120	56
16	1000	40	NA	1.9943	NA	NA	NA	NA	1046	1260	0.1260	63
17	1000	40	0.9728	0.9497	262	601	0.06006	30	498	600	0.0600	30

[a] Acid = Volume of 1:2 HCl–water required to make standard.

[b] Serial dilution from another standard (e.g., 40 of Standard 6 means add 40 mL of Standard 6).

[c] NA = Not applicable.

Table 2015.18F. Calibration criteria for acid-soluble or "total" P and K

Element ID	Wavelength, nm[a]	Calibration range, µg/mL	Standards used (*see* Table **2015.18D**)	Curve fit	Spectral deconvolution
P	213.618 (1)	0 to 245	Blank, 1, 2, 3, 4, 5, 6	Linear	Cu 213.598
P	213.618 (2)	184 to 472	5, 6, 7, 8, 9, 10	Linear	Cu 213.598
P	214.914 (1)	0 to 245	Blank, 1, 2, 3, 4, 5, 6	Linear	Cu 214.898
P	214.914 (2)	184 to 472	5, 6, 7, 8, 9, 10	Linear	Cu 214.898
K	766.485 (1)	0 to 332	Blank, 1, 2, 3, 4, 5, 6, 7	Linear	None
K	766.485 (2)	332 to 1046	11, 12, 13, 14, 15, 16, 17	Linear	None
K	769.897 (1)	0 to 332	Blank, 1, 2, 3, 4, 5, 6, 7	Linear	Possible $LiNO_3$
K	769.897 (2)	332 to 1046	11, 12, 13, 14, 15, 16, 17	Linear	Possible $LiNO_3$

[a] (1) and (2) distinguish between the same wavelength used to cover two separate concentration ranges.

Table 2015.18G. Final ICP-OES conditions utilized for acid-soluble or "total" P and K validation

Factor	Setting
Power, kW	1.15[a]
Plasma flow, L/min	15
Auxiliary flow, L/min	1.5
Nebulizer pressure, L/min	0.40
Nebulizer type	V-grove
Spray chamber	Scott's (baffled)
Sample pump tube	Orange/white (id = 0.64 mm)
Buffer/internal standard pump tube	Orange/white (id = 0.64 mm)
CsCl concentration, M	0.035
Internal standard and concn, µg/mL	6
Buffer matrix	2% nitric acid
Exposure length, s	10
Number of exposures	3
Rinse time, s	30
Total analysis time, min	2.4

[a] A power of 1.20 kW is required for a Thermo 6500 radial vie w.

FOOD BIOLOGICAL CONTAMINANTS

Evaluation of the VITEK® 2 Gram Positive (GP) Microbial Identification Test Card: Collaborative Study

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A collaborative study was conducted to evaluate the performance of the VITEK® 2 Gram Positive (GP) identification card for use with the VITEK 2 automated microbial identification system. The GP test card is used in the identification of selected Gram positive organisms, including *Listeria* and *Staphylococcus* species. The VITEK 2 GP card is based on 43 biochemical tests measuring carbon source utilization, inhibition and resistance, and enzymatic activities. A total of 20 laboratories representing government, industry, and private testing laboratories throughout the United States participated. In this study, 720 Gram-positive inclusivity isolates were analyzed by the GP Identification method. Of the 720 well-characterized isolates, 714 were identified correctly, zero were misidentified, zero were unidentified, and six were not characterized as a Gram-positive organism by the VITEK 2 GP method. Additionally, 120 strains exclusive of Gram-positive organisms were screened by Gram stain. A total of 106 isolates were correctly excluded. Fourteen organisms were incorrectly characterized by Gram stain procedures, thus resulting in improper analysis and misidentification by VITEK GP. The VITEK 2 GP identification method is an acceptable automated method for the rapid identification of selected Gram-positive bacteria.

The rapid and accurate identification of foodborne organisms is a critical component for ensuring the safety of consumers. Traditional methods to detect foodborne bacteria often rely on time-consuming growth in culture media, followed by isolation, biochemical identification, and sometimes serology (1). The VITEK 2® system addresses this need by providing automated testing to identify microorganisms present in raw materials, production environments, and finished products. The technology of the VITEK 2 system enables fast identification, within hours rather than the days required for classical methods (2). Gram-positive organism identification is achieved through the use of the Gram Positive (GP) Identification Card. This card is intended to be used with the VITEK 2 system for the automated identification of most significant Gram-positive organisms. The GP card is a single-use disposable and is based on established biochemical methods and newly developed substrates measuring carbon source utilization, resistance, and enzymatic activities. There are 43 biochemical tests and one negative control well within each GP card. Identification results are available in approximately 8 h or less (3). The identification of the test organism is based on the data and knowledge about the organism and reactions being analyzed. As part of the identification process, the VITEK 2 software compares the test set of reactions to the expected set of reactions of each organism. A qualitative value, referred to as the percent probability of the identification result, is calculated and reported alongside the identification result. This value relates to how well the observed reactions compare to the typical reactions of each organism (4).

The VITEK 2 GP test method was validated according to AOAC guidelines for *Performance Tested Method*SM (PTM) and *Official Method*SM (OMA) precollaborative studies for the identification of *Listeria* and *Staphylococcus* species (5). In the combined contract laboratory and AOAC independent laboratory studies, 63 inclusivity strains representing seven *Listeria* species and four *Staphylococcal* species claims (*Listeria grayi, Listeria innocua, Listeria ivanovii* ssp. *Ivanovii, Listeria ivanovii* ssp. *Londoniensis, Listeria monocytogenes, Listeria seeligeri, Listeria welshimeri, Staphylococcus hyicus, Staphylococcus intermedius, Staphylococcus aureus,* and *Staphylococcus epidermidis*) from the VITEK 2 GP database and 40 exclusivity strains were challenged for correct identification. All test strains were tested from three recommended culture media: Trypticase soy agar (TSA), Columbia agar with 5% sheep blood (CBA), and Trypticase soy agar plates with 5% sheep blood (TSAB) at 12 and 48 h of incubation. Overall percent correct identifications in the combined internal and independent studies from 12 h TSA, CBA, and TSAB cultures were 98, 97, and 98%, respectively. Overall correct identifications in the combined internal and independent studies for all test strains from 48 h TSA, CBA,

Submitted for publication March 1, 2012.
The recommendation was approved by the Methods Committee on Microbiology as First Action. *See* "Standards News," (2012) *Inside Laboratory Management*, March/April issue.
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DOI: 10.5740/jaoacint.CS2012_02

Table 1. Inclusivity (claimed) isolates used in VITEK 2 GP collaborative study

Organisms	ID No.	Source[a]	Origin
Listeria grayi	125274	BMX	Industry
Listeria innocua	12524	BMX	Fish
Listeria ivanovii ssp. *Ivanovii*	12913	BMX	Reference lab
Listeria ivanovii ssp. *Londoniensis*	12914	BMX	Clinical
Listeria monocytogenes	12502	BMX	Shellfish
Listeria seeligeri	6217	BMX	Creamer
Listeria welshimeri	12517	BMX	Beef
Staphylococcus hyicus	13889	BMX	Reference lab
Staphylococcus intermedius	16409	BMX	Veterinary
Staphylococcus aureus	8819	BMX	Reference lab
Staphylococcus aureus	8852	BMX	Reference lab
Staphylococcus epidermidis	8890	BMX	Reference lab

[a] BMX = bioMérieux culture collection.

and TSAB cultures were 95, 96, and 97%, respectively. The VITEK 2 GP reagents were stable over 18-month time studies and provided highly reproducible results when compared between lots. Assay ruggedness was demonstrated for three critical parameters, including age of culture, inoculum density ,and inoculum age. Overall results indicate that the VITEK 2 GP method is an acceptable automated method for the identification of selected Gram-positive organisms. The method was awarded PTM certification No. 120702 on December 10, 2007.

The collaborative study evaluated the ability of the VITEK 2 GP identification method to correctly identify 720 inclusivity strains representative of 12 different claimed organisms. A total of 120 exclusivity strains representative of six different nonclaimed organisms were also evaluated. All isolates analyzed were obtained from the three recommended subculture culture media: TSA, CBA, and TSAB (6).

Collaborative Study

Study Design

A total of 20 laboratories participated in the collaborative study, consisting of industry, government, and private testing laboratories involved in the testing of food products. Qualified laboratories currently using the VITEK 2 system were invited to act as collaborators. Each participating laboratory received 18 isolates on Brain Heart Infusion (BHI) agar slants. The identity of each isolate had been previously confirmed by traditional biochemical confirmations and/or Certificate of Analysis. There were 12 claimed isolates (target inclusivity organisms; Table 1) and six isolates of exclusivity organisms (nontarget organisms; Table 2).

A detailed collaborative study packet outlining all necessary information related to the study, including isolate preparation and documentation of results, was sent to each collaborating laboratory prior to the initiation of the study. All participating laboratories were provided with GP cards, prepared culture media plates, and any additional supplies needed to identify the isolates. Participants were asked to provide their own Gram stain reagents due to challenges associated with federal regulations in the transportation of the reagents.

Preparation of Isolates

Pure isolates of each organism from the bioMérieux (BMX) culture collection were sent on BHI agar slants to each of the participating laboratories. Each isolate used had been well characterized. Microscopic and plate morphology, standard biochemicals, at least two phenotypic methods, and, when needed, molecular sequencing were performed. To prepare for distribution to the collaborators, growth from each strain was transferred to BHI agar slants from growth that had been subcultured onto CBA and incubated for 18–24 h at 35–37°C. Following incubation, slants were labeled and prepared for shipment.

Isolate Distribution

All isolates were labeled with a randomized, blind-coded 3-digit number affixed to the sample vial. Isolates were shipped in leak-proof, insulated containers on a Monday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transportation Association. Upon receipt on Tuesday, isolates were streaked onto CBA to check for purity. Collaborators were instructed to hold the original BHI agar slants in the refrigerator at 2–5°C for the duration of the study. All isolates were shipped at ambient temperature. Collaborators received all 18 isolates during the same week of testing. Participants were instructed to inspect the isolates upon receipt of the package and document that they were received in good condition on the Sample Receipt Confirmation form provided in the shipment. Forms were then faxed or emailed to the study director.

Analysis of Isolates

Upon receipt of the shipment, collaborators were instructed to streak each isolate received onto CBA to check for purity and incubate for 18–24 h at 35–37°C. Following incubation, a Gram stain was conducted on one isolated colony from each strain according to the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (FDA/BAM) method (7) and results were recorded. Those isolates that were identified as characteristic Gram-positive organisms were streaked to the three recommended culture media, TSA, CBA, and TSAB, and

Table 2. Exclusivity isolates used in VITEK 2 GP collaborative study

Organisms	ID No.	Source[a]	Origin
Bacillus coagulans	202608	BMX	Tomato paste
Citrobacter freundii	110125	BMX	Hospital
Escherichia coli	112380	BMX	Beef hide
Serratia marcescens	111663	BMX	Reference lab
Aspergillus niger	304232	BMX	Clinical
Candida albicans	305527	BMX	Reference lab

[a] BMX = bioMérieux culture collection.

Table 2012.02A. Interlaboratory study results for the VITEK 2 GP identification method: Claimed isolates

Organism	Correct	Misidentified	Unidentified	Not tested[a]	Total[b]
Listeria grayi	60	0	0	0	60
Listeria innocua	60	0	0	0	60
Listeria ivanovii ssp. *Ivanovii*	60	0	0	0	60
Listeria ivanovii ssp. *Londoniensis*	60	0	0	0	60
Listeria monocytogenes	60	0	0	0	60
Listeria seeligeri	57	0	0	3	60
Listeria welshimeri	60	0	0	0	60
Staphylococcus hyicus	60	0	0	0	60
Staphylococcus intermedius	60	0	0	0	60
Staphylococcus aureus	60	0	0	0	60
Staphylococcus aureus	57	0	0	3	60
Staphylococcus epidermidis	60	0	0	0	60
Total isolates	714	0	0	6	720

[a] Organism was incorrectly characterized by Gram stain and was not tested on VITEK 2 GP card.

[b] Total numbers represent isolates analyzed on the three recommended culture media: CBA, TSA, and TSAB.

incubated at 35–37°C aerobically for 18–24 h. From each of the incubated plates, a culture suspension was prepared and the organisms were identified as described in the method. Printed results indicated a high probability match to a single species if a unique identification pattern was recognized. If a unique pattern was not recognized, the system suggested supplemental tests to distinguish between two or three closely related organisms, or indicated the result as an unidentified organism. As indicated in the VITEK 2 GP product information provided to end-users, slashline or low discrimination identifications were considered acceptable results for the VITEK 2 GP method that required supplemental tests to further resolve the organism identification. Results were classified as I, Identified; UD, Unidentified; and MI, Misidentified. The results of each isolate identification were recorded on the data reporting sheets provided and were sent along with the VITEK 2 printouts to the study director within 2 weeks of completion of the study. No additional testing was required for isolates not characterized as Gram-positive organisms by Gram stain procedures.

AOAC Official Method 2012.02
Gram-Positive Bacteria Identification
VITEK® 2 Gram Positive (GP)
Biochemical Identification Method
First Action 2012

(Applicable to the identification of Gram-positive bacteria, including *Listeria* and *Staphylococcus* species.)

See Tables **2012.02A** and **2012.02B** for results of the interlaboratory study supporting acceptance of the method.

Claimed Gram-positive species validated in both the precollaborative and collaborative studies: *Listeria grayi, Listeria innocua, Listeria ivanovii* ssp. *Ivanovii, Listeria ivanovii* ssp. *Londoniensis, Listeria monocytogenes, Listeria seeligeri, Listeria welshimeri, Staphylococcus hyicus, Staphylococcus intermedius, Staphylococcus aureus, Staphylococcus aureus,* and *Staphylococcus epidermidis*.

A. Principle

The VITEK 2 system is an automated microbial identification system that utilizes the VITEK 2 Gram-positive (GP) identification card for the identification of most significant Gram-positive organisms. The VITEK 2 GP card is based on 43 biochemical tests measuring carbon source utilization, inhibition and resistance, and enzymatic activities. Identification results are available in approximately 8 h or less.

B. Apparatus and Reagents

(**a**) *VITEK 2 system.*
(**b**) *VITEK 2 GP test cards.*
(**c**) *Vortex.*
(**d**) *Incubators*.—Set to 30°C and 35–37°C.
(**e**) *VITEK 2 DENSICHEK kit.*
(**f**) *DENSICHEK calibrator*.
(**g**) *VITEK 2 cassette*.
(**h**) *Sterile saline*.—Aqueous 0.45 to 0.50% NaCl, pH 4.5 to 7.0).
(**i**) *Disposable test tubes*.—12 mm × 75 mm clear plastic (polystyrene).
(**j**) *Sterile sticks or swabs.*
(**k**) *Brain Heart Infusion (BHI) slants.*
(**l**) *Culture media*.—Columbia agar with 5% sheep blood (CBA), Trypticase soy agar (TSA), or Trypticase soy agar with 5% sheep blood (TSAB) plates.

C. General Instructions

(**a**) The test cannot be performed without a pure culture. A pure culture may be obtained by streaking out the isolate on CBA, TSA, or TSAB plates.
(**b**) Gram stain purified isolate prior to preparing VITEK 2 GP test card to verify presence of Gram-positive organism.
(**c**) *Caution*: Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. All microbial cultures are potentially infectious and should be treated with universal precautions.

Table 2012.02B. Interlaboratory study results for the VITEK 2 GP identification method: Exclusivity isolates

Organism	Misidentified[a]	Unidentified[b]	Not tested[c]	Total[d]
Bacillus coagulans	3	27	10	40
Citrobacter freundii	0	0	20	20
Escherichia coli	0	6	14	20
Serratia marcescens	0	0	20	20
Aspergillus niger	0	0	20	20
Candida albicans	6	0	14	20
Total Isolates	9[e]	33[e]	98	140

[a] Organism was incorrectly characterized by Gram stain and was improperly tested by the VITEK 2 GP method resulting in misidentification.

[b] Organism was incorrectly characterized by Gram stain and was improperly tested by the VITEK 2 GP method resulting in no identification.

[c] Organism was excluded by Gram stain procedure and was not tested on VITEK 2 GP card as per protocol.

[d] Total numbers represent each strain not tested and strains that were misidentified and unidentified.

[e] Total number of isolates incorrectly tested by the VITEK 2 GP method after erroneous Gram stain result.

(**d**) Store VITEK 2 GP cards at 2–8°C.

(**e**) Do not freeze test cards.

(**f**) Bring reagents to room temperature before inserting them into the VITEK 2 instrument.

(**g**) Return unused cards to 2–8°C immediately after use.

Note: A Gram stain should be performed to determine a pure culture's Gram reaction and morphology prior to selecting which VITEK 2 identification card to inoculate. Interpretation of test results requires the judgment and skill of a person proficient in Gram staining and knowledgeable in the interpretation of the Gram reaction and morphology of microorganisms.

D. Preparation of Test Suspension

(**a**) Aseptically transfer 3.0 mL sterile saline (aqueous 0.45 to 0.50% NaCl, pH 4.5–7.0) into polystyrene test tubes (12 × 75 mm). Do not use glass tubes.

(**b**) Using a sterile stick or swab, transfer a sufficient number of colonies from a 24 h culture on recommended culture medium to the saline tube to achieve a density equivalent to McFarland 0.50 to 0.63 with the VITEK 2 DENSICHEK.

(**c**) Test the cultures by the VITEK 2 GP method within 30 min of preparation of the suspended culture.

(**d**) Insert the culture tube and the VITEK 2 GP card into the VITEK 2 cassette and refer to the User Manual (to be provided with the instrument) for instructions on use of the instrument.

(**e**) Report identification results from the VITEK 2 system.

(**f**) As indicated in the VITEK 2 GP product information provided to end-users, slashline or low discrimination identifications are acceptable results for the VITEK 2 GP method that require supplemental tests to further resolve the organism identification.

E. Results and Interpretation

The results are interpreted by the VITEK 2 system. Printed results will indicate a high probability match to a single species if a unique identification pattern is recognized. If a unique pattern

Table 2012.02C. Biochemical tests included in the VITEK 2 GP card

Well	Test	Abbreviation
2	D-Amygdalin	AMY
4	Phosphatidylinositol phospholipase C	PIPLC
5	D-Xylose	dXYL
8	Arginine Dihydrolase 1	ADH1
9	β-Galactosidase	BGAL
11	α-Glucosidase	AGLU
13	Ala Phe Pro arylamidase	APPA
14	Cyclodextrin	CDEX
15	L-Aspartate arylamidase	AspA
16	β Galactopyranosidase	BGAR
17	α-Mannosidase	AMAN
19	Phosphatase	PHOS
20	Leucine arylamidase	LeuA
23	L-Proline arylamidase	ProA
24	β-Glucaronidase	BGURr
25	α-Galactosidase	AGAL
26	L-Pyrrolidonyl-arylamidase	PyrA
27	β-Glucaronidase	BGUR
28	Alanine arylamidase	AlaA
29	Tyrosine arylamidase	TyrA
30	D-Sorbitol	dSOR
31	Urease	URE
32	Polymixin B resistance	POLYB
37	D-Galactose	dGAL
38	D-Ribose	dRIB
39	L-Lactate alkalinization	ILATk
42	Lactose	LAC
44	*N*-Acetyl-D-glucosamine	NAG
45	D-Maltose	dMAL
46	Bacitracin resistance	BACI
47	Novobiocin resistance	NOVO
50	Growth in 6.5% NaCl	NC6.5
52	D-Mannitol	dMAN
53	D-Mannose	dMNE
54	Methyl-B-D-glucopyranoside	MBdG
56	Pullulan	PUL
57	D-Raffinose	dRAF
58	O/129 Resistance (comp.vibrio.)	O129R
59	Salicin	SAL
60	Saccharose/sucrose	SAC
62	D-Trehalose	dTRE
63	Arginine dihydrolase 2	ADH2s
64	Optochin resistance	OPTO

is not recognized, the system will suggest supplemental tests to distinguish between two or three closely related organisms, or indicate the result as an unidentified organism (either >3 organisms can exhibit the observed pattern, or the biopattern is very atypical and is not represented in the database). It is recommended that hemolysis on blood agar is reviewed for any identification of *Listeria innocua.* If β-hemolysis is observed, further testing must be performed to exclude *Listeria monocytogenes*.

Reference: *J. AOAC Int.* **95**, 1427(2012)

Results

In the collaborative study, a total of 720 isolates were analyzed representing 12 different inclusivity species and 120 isolates screened representing six different exclusivity species. Twenty laboratories representing government, industry, and private testing laboratories throughout the United States participated. The individual isolate results for both inclusivity and exclusivity reported by each laboratory are presented in Tables 3 and 4, respectively. Tables **2011.02A** and **2011.02B** present a summary of the collaborative study including the number of isolates identified correctly, misidentified, unidentified, or not tested. For the purposes of this collaborative study, an unidentified or misidentified isolate was considered a missed identification.

Discussion

Inclusivity

For the inclusivity evaluation, each laboratory identified isolates from CBA, TSA, and TSAB subculture media for each of the 12 claimed species. All of the collaborators completed all of the isolates submitted. A total of 714 strains were identified correctly by the VITEK 2 GP method. Six isolates were not tested by the VITEK 2 GP method due to being excluded by Gram stain reactions. For *L. innocua*, Laboratory 3 reported a low discrimination between *L. innocua* and *L. welshimeri* on TSA only. Laboratories 2, 3, 11, and 13 reported low discrimination between *L. seeligeri, L. ivanovii,* and *L. welshimeri* on either CBA or TSAB for *L. seeligeri*. Laboratories 6, 10, 11, and 14 reported low discrimination between *L. innocua* and *L. welshimeri* for *L. welshimeri* on TSA or TSAB. Laboratories 10, 11, and 18 reported low discrimination between *S. aureus* and *S. intermedius* for *S. aureus* on TSAB only. As indicated in the VITEK 2 GP method, a low discrimination result is considered a correct identification. Supplemental tests recommended on the result printout were conducted to provide a correct final identification of the species. There were zero isolates that produced a misidentified or an unidentified result for the inclusivity panel.

Exclusivity

For the exclusivity evaluation, each laboratory received six nontarget species that consisted of three Gram-negative bacteria, one *Bacillus*, one yeast, and one mold. All of the collaborators completed all of the isolates submitted. Of the 120 isolates screened, 106 were correctly excluded. A total of 14 isolates were incorrectly characterized as a Gram-positive organism resulting in improper analysis and misidentification by VITEK 2 GP. Laboratories 1, 2, 5, 7, 8, 10, 11, 15, and 18 characterized *B. coagulans* as a Gram-positive or a Gram-positive with endospores and resulted in an Unidentified Organism reported by the VITEK 2 GP method. This is due to the fact that Bacilli are not covered in the scope of the VITEK 2 GP test system. Laboratory 13 characterized *B. coagulans* as Gram-positive cocci and reported the VITEK 2 GP results as *Streptococcus mitis*. Laboratories 10 and 14 characterized *Escherichia coli* as a Gram-positive organism and reported the VITEK 2 identification as Unidentified Organism. Laboratories 1 and 8 incorrectly characterized *Candida albicans* as a Gram-positive organism and reported the VITEK 2 identification as *Kocuria varians*.

Recommendations

It is recommended that the VITEK 2 GP test card method be adopted as Official First Action for the identification of selected Gram-positive bacterium.

Acknowledgements

We would like to extend a sincere thank you to the following collaborators for their dedicated participation in this study:

John Mills, Judith Colón-Reveles, and Hari P. Dwivedi, bioMérieux Industry, Hazelwood, MO

Yvonne Salfinger, Sun Kim, Patricia Hanson, and Jason Crowe, Florida Department of Agriculture and Consumer Services, Tallahassee, FL

Tom Sidebottom and Carol Elems, FDA San Francisco District Office, Alameda, CA

Maureen Coakley, Jennifer Canale, and Gloria Parra, FDA Northeast Regional Laboratory, Jamaica, NY

Sung Guk Kim and Latriana Robertson, FDA National Center for Toxicological Research, Jefferson, AR

Hua Wang, FDA Center for Food Safety and Applied Nutrition, College Park, MD

Leeann Johnson and Bryanne Shaw, Minnesota Department of Agriculture, St. Paul, MN

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Pete Dombroski, Illinois Department of Public Health Laboratory Springfield Combined Laboratory Facility, Springfield, IL

Mary Ann Murphy, Jackie Glover, and Rebecca Daugherty, U.S. Department of Agriculture-Agricultural Marketing Service, National Science Laboratory, Gastonia, NC

Karen Wilson and Sheri Robeson, Michigan Department of Agriculture, Lansing, MI

Adam Miller, Rhode Island Department of Health Laboratories, Providence, RI

Jessica Dyer and Urvashi Patel, North Carolina Department of Agriculture, Food and Drug Protection Division, Raleigh, NC

Table 3. Inclusivity panel results[a]

Organism	ID No.	Gram reaction	Culture medium[b]	Collaborating laboratory																		
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Listeria grayi	125274	Gram-positive rods	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Listeria innocua	12524	Gram-positive rods	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	LD[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Listeria ivanovii ssp. *Ivanovii*	12913	Gram-positive rods	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Listeria ivanovii ssp. *Londoniensis*	12914	Gram-positive rods	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Listeria monocytogenes	12502	Gram-positive rods	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Listeria seeligeri	6217	Gram-positive rods	CBA	+	+	LD[d]	NT[e]	+	+	+	+	+	+	LD[d]	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	LD[d]	+	NT	+	+	+	+	+	+	+	+	LD[d]	+	+	+	+	+	+	+
Listeria welshimeri	12517	Gram-positive rods	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	LD[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	LD[d]	LD[d]	+	+	LD[d]	+	+	+	+	+	+	+
Staphylococcus hyicus	13889	Gram-positive cocci	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus intermedius	16409	Gram-positive cocci	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus	8819	Gram-positive cocci	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	LD[f]	LD[f]	+	+	+	+	+	+	LD[f]	+	+
Staphylococcus aureus	8852	Gram-positive cocci	CBA	+	+	+	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	NT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus epidermidis	8890	Gram-positive cocci	CBA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			TSAB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] + = Culture was correctly identified by the collaborating laboratory.

[b] CBA = Columbia agar with 5% sheep blood, TSA = Trypticase soy agar, TSAB = Trypticase soy agar with 5% sheep blood.

[c] Low discrimination between *L. innocua/L.welshimeri* reported by VITEK 2; supplemental tests were conducted to report final identification.

[d] Low discrimination between *L.seeligeri/ivanovii/welshimeri* reported by VITEK 2; supplemental tests were conducted to report final identification.

[e] NT = Not tested. Isolate was not identified as Gram positive and thus not tested on the VITEK 2 GP card.

[f] Low discrimination between *S. aureus/S. intermedius* reported by VITEK 2; supplemental tests were conducted to report final identification.

Table 4. Exclusivity panel results[a]

Organism	ID No.	Gram reaction	Culture medium[b]	Collaborating laboratory																		
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Bacillus coagulans	202608	Gram-positive rods with endospores	CBA	UI[c]	UI[c]	–	–	UI[c]	–	UI[c]	UI[c]	–	UI[c]	UI[c]	–	MI[d]	–	UI[c]	–	UI[c]	–	–	–
			TSA	UI[c]	UI[c]	–	–	UI[c]	–	UI[c]	UI[c]	–	UI[c]	UI[c]	–	MI[d]	–	UI[c]	–	UI[c]	–	–	–
			TSAB	UI[c]	UI[c]	–	–	UI[c]	–	UI[c]	UI[c]	–	UI[c]	UI[c]	–	MI[d]	–	UI[c]	–	UI[c]	–	–	–
Citrobacter freundii	110125	Gram-negative rods	CBA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			TSA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			TSAB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Escherichia coli	112380	Gram-negative rods	CBA	–	–	–	–	–	–	–	–	–	UI[e]	–	–	–	UI[e]	–	–	–	–	–	–
			TSA	–	–	–	–	–	–	–	–	–	UI[e]	–	–	–	UI[e]	–	–	–	–	–	–
			TSAB	–	–	–	–	–	–	–	–	–	UI[e]	–	–	–	UI[e]	–	–	–	–	–	–
Serratia marcescens	111663	Gram-negative rods	CBA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			TSA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			TSAB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Aspergillus niger	304232	Hyphae	CBA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			TSA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			TSAB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Candida albicans	305527	Yeast	CBA	MI[f]	–	–	–	–	–	–	MI[f]	–	–	–	–	–	–	–	–	–	–	–	–
			TSA	MI[f]	–	–	–	–	–	–	MI[f]	–	–	–	–	–	–	–	–	–	–	–	–
			TSAB	MI[f]	–	–	–	–	–	–	MI[f]	–	–	–	–	–	–	–	–	–	–	–	–

[a] – = Culture was correctly screened as an exclusive organism and was not tested by the VITEK 2 GP method.

[b] BA = Columbia agar with 5% sheep blood, TSA = Trypticase soy agar, TSAB = Trypticase soy agar with 5% sheep blood.

[c] UI = Unidentified organism. Organism was incorrectly screened by Gram stain procedures and tested by VITEK 2 GP, which is not inclusive of Bacilli.

[d] MI = Misidentified as *Streptococcus mitis* after organism was incorrectly screened by Gram stain procedures and tested by VITEK 2 GP, which is not inclusive of Bacilli.

[e] UI = Unidentified organism. Organism was incorrectly screened as a Gram positive rod by Gram stain procedures and tested by VITEK 2 GP.

[f] MI = Misidentified as *Kocuria varians*. Organism was incorrectly screened by Gram stain procedures and tested by VITEK 2 GP.

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Total Iodine in Infant Formula and Adult/Pediatric Nutritional Formula by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS): Collaborative Study, Final Action 2012.15

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A collaborative study was conducted to determine total iodine in infant formula and adult/pediatric nutritional formula by inductively coupled plasma-MS (ICP-MS) using AOAC First Action *Official Method*SM 2012.15. The purpose of this study was to evaluate the method's intralaboratory and interlaboratory performance and submit the results to AOAC INTERNATIONAL for adoption as a Final Action *Official Method* for the determination of total iodine in infant formula and adult/pediatric nutritional formula. Upon providing acceptable results for practice samples National Institute of Standard and Technology (NIST) Standard Reference Material (SRM) 1849a and a low-fat adult nutritional powder, 13 laboratories analyzed seven various infant and adult nutritional products including a blind duplicate of each. Products were chosen with varying levels of iodine and included low-fat, soy-based, and milk-based formulas and NIST SRM 1849a. Random identification numbers were assigned to each of the seven fortified test materials. Digestion of the test samples occurred using a potassium hydroxide solution in an oven or open-vessel microwave system. Iodine was stabilized with ammonium hydroxide and sodium thiosulfate after digestion. The solutions were brought to volume followed by filtration. The filtrates were then analyzed by ICP-MS after dilution. Results for all seven test samples met all the AOAC *Standard Method Performance Requirements* (SMPR® 2012.008) guidelines. The RSD_r ranged from 0.77 to 4.78% and the RSD_R from 5.42 to 11.5%. The Horwitz ratio (HorRat) for each result was excellent, ranging from 0.35 to 1.31%. The results demonstrate that the method is fit-for-purpose to determine iodine in infant formula and adult/pediatric nutritional formula.

Iodine plays a very important role in maintaining a healthy thyroid gland in humans. Hormones produced by the thyroid are essential for ensuring a healthy body. Benefits include maintaining appropriate metabolism and reproductive function. Perhaps the most critical time for regulation of thyroid hormone production is prenatal, infancy, and childhood when proper growth and development is imperative. Several sources providing optimal amounts of iodine to ensure a well-functioning thyroid gland include fortified infant, pediatric, and adult nutritional formulas. Due to the nutritional benefits provided by iodine, a method for accurate quantification of iodine in these products is of the utmost importance (1).

While a matrix-focused method (AOAC *Official Method*SM **992.24** *Iodide in Ready-to-Feed Milk-Based Infant Formula, Ion-Selective Electrode*) was available, a dispute resolution method capable of very low and accurate determination of iodine in a variety of infant and adult/pediatric nutritional formula was needed. In 2012 the AOAC Expert Review Panel (ERP) on Nutrient Methods approved and assigned First Action status for AOAC INTERNATIONAL *Official Method* **2012.15** (2). In August 2013, based on the results of a single-laboratory validation (SLV; 3), AOAC *Official Method* **2012.15** was chosen by the AOAC ERP as the most appropriate method for the determination of total iodine in infant formula and adult/pediatric nutritional formula to be subjected to a full collaborative study in 2014. Upon successful completion and review of the data, in March 2015 the AOAC ERP approved AOAC *Official Method* **2012.15** for Final Action.

Received February 23, 2015. Accepted by SG April 7, 2015.
The method was approved by the AOAC Official Methods Board as Final Action. *See* "Standards News," (2015) *Inside Laboratory Management*, July/August issue.
The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) invites method users to provide feedback on the Final Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author.
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DOI: 10.5740/jaoacint.15141

Collaborative Study

Invitations to participate in the collaborative study of AOAC First Action *Official Method* **2012.15** were sent to 38 laboratories. Twenty-four laboratories expressed interest in participating. Qualification samples were sent to 20 laboratories after four laboratories made the decision not to participate for various reasons. Six laboratories did not meet acceptance criteria. The remaining 14 laboratories went on to analyze seven test samples (13 laboratories submitted test sample data). Test samples used in this study were obtained from commercial sources and provided by AOAC INTERNATIONAL.

Upon successful completion of two qualification samples, individually prepared test kits, including seven test samples and their blind duplicates, were provided to each collaborator. All powdered samples, with the exception of National Institute of Standards and Technology Standard Reference Material (NIST SRM) 1849a, were required to be analyzed on a reconstituted basis where approximately 25 g of material was diluted with approximately 200 g of deionized water resulting in a total weight of approximately 225 g. Once the test sample was in solution and well mixed, an accurately weighed aliquot of approximately 6 or 12 g (depending on final transfer volume) was subsampled (while continuously stirring) for analysis. This reconstituted solution was discarded after 24 h. Approximately 0.5 or 1 g (depending on final transfer volume) of the NIST SRM 1849a was weighed for analysis. For ready-to-feed (RTF) samples, the laboratory weighed approximately 1 or 2 g (depending on final transfer volume) for analysis. The remaining RTF solutions were transferred to a sealed, brown polypropylene container and held at refrigerated conditions between 2 and 8°C. These solutions were discarded after 5 days.

The test samples were shipped at ambient temperature. Collaborators were asked to store the samples at room temperature before and during analysis with the exception of the RTF samples, which were refrigerated after the initial sampling.

Bulk standards were to be stored as directed on the certificate of analysis/receipt paperwork. Laboratories were directed to follow instructions in the method for storage and shelf life of solutions.

Once analysis of the test samples was successfully completed, study participants were asked to complete and submit a spreadsheet summarizing an abundance of information, including (but not limited to) aliquot (sample weight subjected to analysis), digestion technique used, oven or microwave used, instrument make/model used, solution preparation codes, curve information, analysis batch codes, checklist of 10 different QC/study checks, and results as µg/100 g reconstituted final product. Study participants were asked to record comments (positive or negative) and to provide deviations (if any) from the protocol.

All test sample data were subjected to statistical analysis per AOAC requirements, which included overall average, RSD_r, RSD_R, and Horwitz ratio (HorRat). Cochran's maximum variance ratio test (2.5% significance level) and Grubbs' outlier test (single and double, 2.5% significance level) were used to determine outliers.

The method protocol sent to the collaborating laboratories was as described in AOAC First Action Method **2012.15** but with a significantly greater amount of detail. The method below appears as presented in the protocol but now includes improvements and/or additional information as suggested by the AOAC ERP. It also includes minor modifications taken from comments provided by several collaborators, as well as incorporation of components requiring clarification as suggested by the Study Director.

AOAC Official Method 2012.15
Determination of Total Iodine in Infant Formula and Adult/Pediatric Nutritional Formula
Inductively Coupled Plasma-MS (ICP-MS)
First Action 2012
Final Action 2015

[Applicable to the measurement of total iodine in infant formula and adult/pediatric nutritional formula from 0.5 to 1500 µg/100 g reconstituted final product and for RTF products from 2.5 to 1000 µg/100 g using ICP-MS. This method is not applicable to products containing FD&C Red Dye No. 3 (erythrosine). The iodine from erythrosine is also quantitatively determined by this method; thus, accurate quantification of fortified levels of iodine is not possible.]

See Table **2012.15A** for results of the interlaboratory study supporting acceptance of the method.

Caution: Refer to Material Safety Data Sheets (MSDS) for safety precautions when using chemicals. Use personal protective equipment recommended in MSDS.

A. Principle

Digestion occurs using a potassium hydroxide (KOH) solution in an oven or open-vessel microwave system. Iodine is stabilized with ammonium hydroxide and sodium thiosulfate after digestion. The solution is brought to volume followed by filtration. The filtrate is analyzed directly or after dilution by ICP-MS.

B. Safety Considerations

(a) Use only ovens and microwave ovens specifically designed for laboratory use.

(b) The method involves the use of strong bases and concentrated acids. Avoid spills, inhalation, and exposure to human tissues.

(c) Oven and microwave digestion procedures involve moderately elevated temperatures. Carefully remove samples and allow cooling before removing the lids from the digestion vessels.

C. Chemicals and Reagents

(a) *KOH pellets.*—Certified ACS grade (Fisher Scientific, Fairlawn, NJ). (*Note*: KOH may contribute background levels of iodine.)

(b) *Ammonium hydroxide 28–30% (NH_4OH).*—Certified ACS PLUS (Fisher Scientific).

(c) *Sodium thiosulfate ($Na_2S_2O_3$).*—≥99.99% metal basis (Fisher Scientific).

(d) *Surfactant (e.g., Triton® X-100).*—Sigma (St. Louis, MO).

Table 2012.15A. Statistical data

Sample name	Average	S_r[a]	RSD_r	S_R[b]	RSD_R	No. of outlier laboratories[c]	HorRat	No. of laboratories used
NIST SRM 1849a, mg/kg	1.24	0.010	0.77	0.067	5.42	1	0.35	12
Infant formula RTF, milk based-1[d]	5.48	0.262	4.78	0.507	9.25	0	1.06	13
Infant formula powder, soy based [d]	12.4	0.313	2.53	0.945	7.62	2	0.98	11
Infant formula powder, milk based[d]	18.5	0.693	3.75	1.39	7.54	2	1.03	11
Infant formula RTF, milk based-2[d]	5.45	0.226	4.16	0.626	11.5	0	1.31	13
Child formula powder [d]	3.47	0.135	3.87	0.278	8.01	2	0.85	11
Adult nutritional powder, low fat[d]	7.03	0.137	1.94	0.503	7.15	2	0.85	11

[a] S_r = SD for repeatability.

[b] S_R = SD for reproducibility.

[c] Values from laboratories with outliers were not used in statistical calculations.

[d] Results expressed as µg/100 g reconstituted final product.

(e) *Nitric acid concentrated (HNO_3).*—OPTIMA (high purity; Fisher Scientific).

(f) *Perchloric acid 70% ($HClO_4$).*—Reagent ACS (Fisher Scientific).

(g) *Purified water.*—18 MΩ/cm.

Note: Equivalent chemicals and reagents may be substituted.

D. Apparatus

(a) *Polypropylene (PP) tubes.*—Assorted sizes, use as received; 50 mL PP DigiTUBES® (Part No. 010-500-261), 100 mL PP DigiTUBES (Part No. 010-501-263); SCP Science (Montreal, Canada).

(b) *Oven (i.e., warming/drying oven).*—Isotemp oven Model 6921 (Fisher Scientific).

(c) *Open-vessel microwave digestion unit (optional).*—MARS 5 or MARS 6 (CEM Corp., Matthews, NC).

(d) *Analytical and top-loader balances.*—Sensitive to 0.0001 and 0.01 g, respectively (Sartorius, Goettingen, Germany).

(e) *ICP-MS system.*—ELAN DRC II (PerkinElmer, Waltham, MA).

(f) *Autosampler for ICP-MS.*—SC4-DX (Elemental Scientific, Inc., Omaha, NE).

(g) *Adjustable (electronic or manual) volumetric pipets.*—Eppendorf (Hamburg, Germany). Capable of volumes 100–5000 µL.

(h) *Re-pipet volumetric dispensers.*—Adjustable volume.

(i) *PP or Teflon bottles for storage of reagents.*

(j) *Disposable plastic syringes.*—e.g., 10 mL with LuerLok.

(k) *Syringe filters with 1 µm membrane.*—Non-sterile glass fiber B (Part No. SLPBDZ5NK; EMD Millipore, Corp., Billerica, MA).

(l) *Beakers.*—Assorted sizes.

(m) *Stir bars.*—7.9 × 50 mm, assorted sizes (VWR, Chester, PA).

(n) *Stir plate.*—Adjustable speed, Corning (Corning, NY) or equivalent.

(o) *Pump tubing.*—Peristaltic, black/black two-stop polyvinyl chloride (PVC), 0.76 mm id (SCP Science, Champlain, NY), used for introducing carrier solution.

(p) *Pump tubing.*—Peristaltic, orange/green two-stop PVC pump tubing, 0.38 mm id (SCP Science), used for introducing internal standard (IS) solution.

Notes: Equivalent apparatus may be substituted.

All laboratory plasticware should be single-use whenever possible. If reuse is necessary, wash using 10% HNO_3, then rinse thoroughly with purified water prior to use. When needed, general laboratory acid-washed glassware may also be used.

Filter membranes <1 µm (e.g., 0.25 or 0.45 µm) may be used.

Adherence as close as possible to the recommended ids of the pump tubing is critical. The ratio of the pump tubing id (0.76 mm) used for the carrier solution to the pump tubing id (0.38 nm) used for the IS solution may be used as a guideline (0.76/0.38 = 2). For best performance, the ratio should remain as close to 2 as possible. Vast differences in id between the carrier solution pump tubing and the IS solution (e.g., 1.02/0.19, respectively) may result in poor accuracy.

E. Instrument and Parameters

(a) *Instrument.*—ICP-MS PerkinElmer ELAN DRC II, or equivalent.

(b) *Mode.*—Standard (STD).

(c) *Gas.*—Argon (≥99.998%, high purity).

(d) *Rinse.*—0.1% Triton/1% NH_4OH in purified water.

(e) *Sweeps/readings.*—20.

(f) *Readings/replicate.*—One.

(g) *Replicates.*—Three.

(h) *Nebulizer gas flow.*—Optimized daily.

(i) *Auxiliary gas flow.*—1.2 L/min.

(j) *Plasma gas flow.*—15.00 L/min.

(k) *Lens voltage.*—Optimized daily.

(l) *ICP radio frequency power.*—1500 watts.

(m) *Peristaltic pump.*—Rate optimized.

Notes: Parameters of other manufacturer's instruments may be optimized accordingly to ensure the instrument's minimum daily performance requirements are met.

All analyses must be performed using the STD mode. (Use of a reaction or collision gas is not required or allowed.)

Table 2012.15B. Preparation of intermediate stock standard (ISS) iodine solutions[a]

Iodine standard solution ID	ID of solution used for preparation	Initial iodine concentration, ng/mL	Aliquot volume, mL	Final volume, mL	Final iodine concentration, ng/mL
10000 (ISS)	Stock	1000000	0.5	50	10000
1000 (ISS)	10000 (ISS)	10000	5	50	1000
10.0 (ISS)	1000 (ISS)	1000	0.5	50	10.0

Aliquot the appropriate amount of iodine standard solution into a single use 50 mL DigiTUBE® and add 5 mL of stabilizer concentrate, fill to the 50 mL mark on the tube with water, cap the tube, and then mix thoroughly. The resulting matrix concentration is 1% NH_4OH and 0.1% $Na_2S_2O_3$ in water.

[a] ISS solutions are used for calibration standard preparation and are typically prepared according to the table. The ISS concentrations presented are nominal. Using the stock iodine concentration found on the certificate of analysis, determine the exact concentration of each ISS. The use of an electronic adjustable volume pipet, capable of delivering 100 to 5000 µL, is recommended.

F. Reference Standards

(a) *Iodide 1000 ppm standard solution in H_2O.*—SPEX CertiPrep (Metuchen, NJ.)

(b) *Iodide 1000 ppm standard solution in 1% triethanolamine (TEA).*—Inorganic Venture (Christiansburg, VA.)

Notes: Either stock iodide reference solutions may be used for intermediate and working standard solutions preparation. The remaining source may be used as a continuing calibration verification (CCV) standard.

Equivalent reference standards may be substituted.

"Iodide" may be referred to as "iodine" throughout this method.

G. Internal Standard

Praseodymium 10 ppm standard solution in 5% HNO_3.—Inorganic Ventures.

Notes: Individual values of iodine will be reported for each test sample using praseodymium as the IS. Equivalent stock IS solutions may be substituted.

H. Procedure

(a) *Reagent solutions preparation.—Note*: Prepare all reagent solutions as recommended by either weight/volume (w/v) or volume/volume (v/v). Adjusting for purity and/or concentration is not required.

(*1*) *5% KOH solution.*—Dissolve 25 g KOH pellets in an appropriate amount purified water, then dilute to 500 mL with purified water. This solution may be added using a re-pipet volumetric bottle top dispenser. Store this solution at room temperature. Reagent expires 6 months after preparation date.

(*2*) *Stabilizer concentrate.*—Dissolve 5 g $Na_2S_2O_3$ in an appropriate amount purified water, add 50 mL NH_4OH, then dilute to 500 mL with purified water. The resulting concentration is 10% NH_4OH and 1% $Na_2S_2O_3$ in purified water. Store this solution at room temperature. Reagent expires 6 months after preparation date.

(*3*) *Wash solution (rinse).*—Dissolve 2 g Triton X-100 in an appropriate amount of purified water, add 20 mL NH_4OH, then dilute to 2 L with purified water. The resulting concentration is 1% NH_4OH and 0.1% Triton X-100 in purified water. This solution may be added using a re-pipet volumetric bottle top dispenser. Store this solution at room temperature. Reagent expires 6 months after preparation date.

(*4*) *Diluent.*—Dissolve 10 g KOH pellets and 0.4 g $Na_2S_2O_3$ in an appropriate amount of purified water, add 4 mL NH_4OH, then dilute to 2000 mL with purified water. Store this solution at room temperature. Reagent expires 6 months after preparation date. Alternatively, for a smaller volume, dilute 50 mL 5% KOH and 10 mL stabilizer concentrate to 500 mL with purified

Table 2012.15C. Preparation of calibration standard (CS) iodine and calibration blank (CB) solutions[a]

Iodine standard solution ID	ID of solution used for preparation	Initial iodine concentration, ng/mL	Aliquot volume, mL	Final volume, mL	Final iodine concentration, ng/mL
100 (CS)	1000 (ISS)	1000	5	50	100
50.0 (CS)	1000 (ISS)	1000	2.5	50	50.0
10.0 (CS)	1000 (ISS)	1000	0.5	50	10.0
1.00 (CS)	10.0 (ISS)	10.0	5	50	1.00
0.500 (CS)	10.0 (ISS)	10.0	2.5	50	0.500
0.250 (CS)	10.0 (ISS)	10.0	1.25	50	0.250
Blank (CB)	NA[b]	NA	NA	50	0

Aliquot the appropriate amount of iodine standard solution into a single-use 50 mL DigiTUBE and add 5 mL of 5% KOH and 1 mL of stabilizer concentrate, fill to the 50 mL mark on the tube with water, cap the tube, and then mix thoroughly. The resulting matrix concentration is 0.5% KOH, approximately 0.2% NH_4OH, and approximately 0.02% $Na_2S_2O_3$ in water.

[a] Typical CS standard concentrations are nominally 0.250, 0.500, 1.00, 10.0, 50.0, and 100 ppb iodine and are typically prepared according to the table. The CB is the zero point of the curve. The curve type used, if using a PerkinElmer ICP-MS system with ELAN software, should be linear through zero. If using an Agilent or Thermo ICP-MS system, force the curve through the calibration blank. The calibration curve must have a correlation coefficient (r) of ≥0.998 to be acceptable. Determine the exact concentration of each CS (traceable back to the certificate of analysis) and assign these values to the curve points used to generate final results. The use of an electronic adjustable volume pipet, capable of delivering 100 to 5000 µL, is recommended.

[b] NA = Not applicable.

Table 2012.15D. Preparation of intermediate continuing calibration verification (ICCV) and continuing calibration verification (CCV) iodine solutions and continuing calibration blank (CCB) solution[a]

Iodine standard solution ID	ID of solution used for preparation	Initial iodine concentration, ng/mL	Aliquot volume, mL	Final volume, mL	Final iodine concentration, ng/mL
10000 (ICCV)	Stock	1000000	0.5	50	10000
1000 (ICCV)	10000 (ICCV)	10000	5	50	1000
10.0 (CCV)	1000 (ICCV)	1000	0.5	50	10.0
Blank (CCB)	NA[b]	NA	NA	50	0

Aliquot the appropriate amount of iodine standard solution into a single-use 50 mL DigiTUBE, fill to the 50 mL mark on the tube with diluent, cap the tube, and then mix thoroughly. The resulting matrix concentration is 0.5% KOH, approximately 0.2% NH_4OH, and approximately 0.02% $Na_2S_2O_3$ in water. For the blank (CCB), fill a single-use 50 mL DigiTUBE to the 50 mL mark on the tube with diluent, cap the tube, and then mix thoroughly.

[a] ICCV solutions are used for preparation of the CCV standard solution and are typically prepared according to the table. The ICCV and CCV concentrations presented are nominal. Using the stock iodine concentration found on the certificate of analysis (from the second source), determine the exact concentration of each ICCV. With this information, determine the exact concentration of the CCV standard. The use of an electronic adjustable volume pipet, capable of delivering 100 to 5000 µL, is recommended.

[b] NA = Not applicable.

water. Store this solution at room temperature. Reagent expires 6 months after preparation date.

Note: The resulting concentrations for both preparations are 0.5% KOH, 0.2% NH_4OH, and 0.02% $Na_2S_2O_3$ in purified water.

(*5*) *Conditioning solution*.—Prepare by aliquoting 25 mL 5% KOH solution, then diluting to 250 mL with purified water. This solution is used to prepare the instrument for analysis. The resulting concentration is 0.5% KOH. Store this solution at room temperature. Reagent expires 6 months after preparation date.

(*6*) *Carrier solution*.—Equivalent to the wash solution. The carrier solution is used to deliver the sample solution to the nebulizer through the ICP-MS autosampler introduction system. The carrier solution is introduced via a peristaltic pump using black/black two-stop PVC pump tubing (0.76 mm id). Store this solution at room temperature. Reagent expires 6 months after preparation date.

(b) *Standard solutions preparation*.—*Notes*: Stock solutions are stable until the date indicated on the certificate of analysis. Intermediate, calibration, continuing calibration verification, and IS solutions are stable at room temperature until the earliest expiration date of all components used to prepare the solution.

All calibration standards, continuing calibration verification, continuing calibration blank, and IS solutions are analyzed as prepared. Do not carry these solutions through sample preparation or digestion.

(*1*) *Stock iodine and praseodymium solutions*.—Purchase of stock iodine and praseodymium standard solutions with accompanying certificates of analysis is recommended.

(*2*) *Intermediate stock standard (ISS) iodine solutions*.—Prepare the ISS iodine solutions according to Table **2012.15B**.

(*3*) *Calibration standard (CS) iodine solutions*.—Prepare the solutions according to Table **2012.15C**.

(*4*) *Intermediate continuing calibration verification (ICCV), continuing calibration verification (CCV) iodine solutions, and continuing calibration blank (CCB)*.—Prepare the ICCV, CCV standard solutions, and CCB blank according to Table **2012.15D**.

Note: A CCV must be prepared from a second source stock solution (e.g., purchased from another vendor) other than that used for the CS solutions.

(*5*) *IS solutions*.—Prepare the IS solution according to Table **2012.15E**. The IS concentration typically used for analysis is 30 ppb praseodymium (Pr).

Notes: Ideally, the intensity generated for the IS should be similar to the intensity of iodine standard at the mid-point of the standard curve.

As some ICP-MS instruments provide greater sensitivity, the concentration of Pr may be adjusted accordingly to provide intensities similar to the intensity generated by the 50.0 ppb iodine standard.

(c) *Reconstitution*.—*Note*: All powdered samples, with the exception of NIST SRM 1849a, are required to be analyzed on a reconstituted basis. Do not reconstitute RTF samples.

Accurately weigh approximately 25 g powdered test sample into an appropriate vessel (e.g., 400 mL beaker) and record the weight. Without zeroing the balance, add water to make approximately 225 g. Record the sample + water weight. Place a stir bar in the mixture and stir on a stir plate to form a homogeneous slurry/suspension. Proceed to *Sample preparation* **(d)**.

Note: This reconstituted solution should be discarded after 24 h.

(d) *Sample preparation*.—Weighing (after weighing all materials, proceed to *Addition of reagents* **(e)**).

(*1*) *Reconstituted material*.—Accurately weigh an aliquot of approximately 6 g reconstituted test sample into a 50 mL DigiTUBE® or 12 g into a 100 mL DigiTUBE.

Table 2012.15E. Preparation of internal standard (IS) solution[a]

Standard solution ID	ID of solution used for preparation	Initial concn, ng/mL	Aliquot volume, mL	Final volume, mL	Final concn, ng/mL
30.0 (Pr)	Stock	10000	1.5	500[b]	30.0

[a] The IS concentration typically used for analysis is 30 ppb. The table outlines a typical preparation scheme.

[b] After aliquoting the 10000 ppb Pr into the 500 mL vessel, add approximately 100 mL water, 10 mL HNO_3, 0.5 mL $HClO_4$, 0.05 g Triton® X-100, and then bring to volume with water and mix thoroughly. The resulting concentration is 2% HNO_3, 0.1% $HClO_4$, and 0.01% Triton® X-100 in water.

Table 2012.15F. Open-vessel microwave digestion parameters[a]

Six 50 mL vessels		
Wattage	Power, %	Minutes
400	10	5
400	20	6
400	20	7
Twelve to eighteen 50 mL vessels		
400	25	10
400	40	10
Twenty-four 50 mL vessels		
400	25	10
400	40	10
400	65	10

[a] Microwave used: CEM MARS 5 or CEM MARS 6. *Use caution*: Ensure vessels do not completely seal (bursting hazard) or overheat (as melting may occur). *Note*: Using AOAC Method **2012.15**, the parameters, with the corresponding number of vessels, produced acceptable results for NIST SRM 1849a infant/adult nutritional formula. For each number of vessel's range, if fewer vessels than the minimum are placed in the microwave, overheating may occur resulting in loss of sample or injury. If greater than the suggested number of vessels is placed in the microwave, the digestion may not be complete.

(*2*) *NIST SRM 1849a.*—Accurately weigh approximately 0.5 g NIST SRM 1849a into a 50 mL DigiTUBE or 1 g into a 100 mL DigiTUBE.

(*3*) *RTF material.*—Accurately weigh approximately 1 g of the RTF test sample into a 50 mL DigiTUBE or 2 g into a 100 mL DigiTUBE.

Note: The remaining RTF material should be transferred to a sealed, brown PP container and held at refrigerated conditions between 2 to 8°C. These solutions should be discarded after 5 days.

(*4*) *Blank.*—Designate at least one 50 mL or 100 mL DigiTUBE digestion vessel as the digest blank. The digestion blank(s) should be treated in the same manner as the samples.

(e) *Addition of reagents (after adding all reagents and mixing, proceed to Oven digestion* ***(f)****, or Open vessel microwave digestion* ***(g)****).*—(*1*) *Water.*—Add 10 mL purified water to each 50 mL DigiTUBE or 20 mL to each 100 mL DigiTUBE.

(*2*) *5% KOH.*—Add 5 mL 5% KOH if material was weighed into a 50 mL DigiTUBE or add 10 mL of 5% KOH if material was weighed into a 100 mL DigiTUBE.

(*3*) *Mixing.*—Seal the vessels and swirl or use a vortex apparatus to mix. Avoid inverting as this may allow sample to adhere to the inner walls of the vessel above the level of the digestion solution.

(f) *Oven digestion.*—(*1*) *Digestion/extraction.*—Digest samples in an oven set to maintain 105 ± 5°C until the dissolution of iodine is complete, approximately 1 h.

Notes: The digestion vessels may either be tightened completely or loosened slightly while in the oven.

Carefully swirl by hand each digestion vessel approximately halfway through the digestion/extraction procedure.

(*2*) *Addition of stabilizer.*—After removal of samples from the oven, add 1 mL of stabilizer concentrate to the 50 mL DigiTUBE samples or add 2 mL if material was weighed into a 100 mL DigiTUBE. Allow samples to cool to room temperature.

Note: Alternatively, allow samples to cool to room temperature first, and then add the stabilizer concentrate.

(*3*) *Final volume.*—If 50 or 100 mL vessels were used for digestion, bring samples to a final volume of 50 or 100 mL respectively, with purified water.

(*4*) *Capping/mixing.*—Cap all vessels, and then invert to mix thoroughly.

(g) *Open vessel microwave digestion.*—(*1*) *Digestion/extraction.*—Place the digestion vessels into the carousel of the open-vessel microwave digestion unit. If less than the maximum capacity is to be digested, distribute the vessels evenly throughout the carousel. Digest the samples in the microwave unit until the dissolution of iodine is complete. *See* Table **2012.15F** for suggested open-vessel microwave digestion parameters.

Note: Vessel caps should be loosened slightly (from fully tightened) during the digestion procedure. *Use caution*: Ensure vessels do not completely seal (bursting hazard) or overheat (melting may occur). Alternatively, instead of just loosening the caps, drill small holes (approximately 3 mm) in the caps. This way the caps can be tightened, but venting (thus the "open" vessel) can occur. Caps may be reused after acid washing.

(*2*) *Addition of stabilizer.*—After removal of samples from the oven, add 1 mL stabilizer concentrate to the 50 mL DigiTUBE samples or add 2 mL if material was weighed into a 100 mL DigiTUBE. Allow samples to cool to room temperature.

Note: Alternatively, allow samples to cool to room temperature first, and then add the stabilizer concentrate.

(*3*) *Final volume.*—If 50 or 100 mL vessels were used for digestion, bring samples to a final volume of 50 or 100 mL, respectively, with purified water.

(*4*) *Capping/mixing.*—Cap all vessels, and then invert to mix thoroughly.

(h) *Sample filtering.*—(*1*) *Filtering.*—Filter each sample solution by filling a disposable syringe with the digested sample solution, attach a 1 µm membrane filter, and then filter an adequate amount (e.g., at least 5 mL) into an appropriate vessel (e.g., 15 mL PP centrifuge tube or autosampler vial) to be used for analysis.

Notes: Samples may be difficult to filter. Use of multiple filter membranes may be required. To ease filtration, allow the inverted sample digestates to rest for a period of time (e.g., 1 h) before filtering.

Digested sample solutions may be stored at ambient temperature. Samples may be stored at ambient temperature indefinitely, as long as the results for the applicable digest blank(s) and/or control sample(s) are acceptable when analyzed.

(i) *Sample dilution.*—Aliquot 5 mL of each sample's filtrate into an appropriate volumetric vessel and then bring to a final volume of 10 mL with diluent.

Note: Analyze all samples diluted 5 to 10 mL as directed above.

I. Determination (Instrument and Parameters see Section E)

Notes: All analyses must be performed using the STD mode. (Use of a reaction or collision gas is not required or allowed.)

Prior to conditioning, calibration, and sample analysis,

ensure the instrument is optimized to meet the manufacturer's minimum daily performance requirements.

(a) *Conditioning.*—Condition the ICP-MS sample introduction system. Analyze the conditioning solution while concomitantly introducing IS solution online (e.g., through a mixing block or T) until conditioned (approximately 1 h). The IS solution is introduced via a peristaltic pump using orange/green two-stop PVC pump tubing (0.38 mm id). After conditioning, begin to aspirate carrier solution while continuing to add IS. Analyze samples using ICP-MS. Ensure the wash solution (rinse) is available and ready for use to rinse out the sample lines and introduction system between each analysis.

Notes: If acidic sample solutions are typically analyzed on the ICP-MS system, perform a thorough cleaning of the entire sample introduction system prior to conditioning. Background counts for both iodine and the IS should be relatively stable (e.g., not ascending or descending).

A dedicated set of cones (sampler and skimmer), if possible, is recommended. Analysis of acid-type (e.g., HNO_3) matrixes with the same set of cones used for iodine analysis may increase conditioning time or produce elevated background levels.

Analyzing several (e.g., at least six) digested samples prior to calibration is recommended. Introducing and analyzing actual digested sample solutions increases conditioning efficiency.

Possible additional maintenance: Due the nature of the digestion/extraction solution (i.e., KOH) and the amount of organic material in the sample solutions, additional maintenance may be required (as compared to typical acid matrix digestions/analysis). Lenses in instruments and/or lens stack assemblies may require more frequent cleaning. Once cleaned, a period of reconditioning may be required.

(b) *Calibration.*—In addition to a calibration blank, working standards of 0.250, 0.500, 1.00, 10.0, 50.0, and 100 ppb are used. Calibrate the ICP-MS system using an autosampler or manually.

Notes: The curve type used should be linear, forced through the calibration blank.

All standards must be included in the calibration curve.

The 0.250 ppb signal must be ≥1.5 times the calibration blank signal. Consistent background throughout the entire analytical run is imperative for a successful analysis. This will be evident based on the results obtained for the CCB.

Table 1. Technique used for sample digestion and the make/model of the instrument used for analysis

Laboratory code	Oven	Microwave	Instrument
A	Yes	No	Thermo iCAP Q
B	Yes	No	Thermo iCAP Q
C	Yes	No	Agilent 7700 x
D	Yes	No	Agilent 7500 ce
E	No	Yes	PE Elan DRC-e
F	No	Yes	PE Elan DRC-e
G	Yes	No	PE Elan DRC II
H	Yes	No	PE Nexion 300D
I	Yes	No	Agilent 7500 cx
J	Yes	No	Agilent 7700 x
K	Yes	No	Agilent 7700 x
L	No	Yes	Agilent 7700
M	Yes	No	Agilent 7500 cx

(c) *Sample analysis.*—Analyze a 5 to 10 mL dilution of each digested filtered sample using ICP-MS.

Notes: A 5 to 10 mL dilution is preferable and required in order to achieve a reporting limit of 0.5 µg/100 g as reconstituted final product or the limit of 2.5 µg/100 g for RTF samples.

Diluting the samples reduces the matrix load on the plasma and may reduce frequency of maintenance (e.g., cleaning cones).

For other applications, samples digested with 5% KOH solution may be analyzed directly or diluted (if necessary) so that the iodine concentration will fall within the calibration range. Alternative volume aliquots may be prepared by placing an aliquot of the filtrate into an appropriate volumetric vessel, and then diluting to an appropriate final volume with diluent. Greater dilutions, such as 1 to 18 mL, would achieve a higher upper reporting limit (e.g., 1500 µg/100 g reconstituted final product).

(d) *Data acceptability.*—The calibration curve must include a calibration blank (as a calibration point). The calibration curve must have a correlation coefficient (r) ≥ 0.998 to be acceptable.

The individual back-calculated calibration standard concentrations must be within 90–110% of the theoretical concentrations to be acceptable.

The 0.250 ppb signal must be ≥1.5 times the calibration blank signal. Consistent background throughout the entire analytical run is imperative for a successful analysis. This will be evident based on the results obtained for the CCB.

A CCB is analyzed after calibration, at least every 10 samples, and after the last sample in the analysis batch to monitor background. A CCB should be of the same matrix as the standards used for calibration. Iodine levels ≤30% of the lowest calibration standard are considered acceptable.

With each batch of samples, at least one digest blank should be prepared in the same manner as the samples. An iodine result of ≤30% of the lowest calibration standard is considered acceptable.

A CCV standard solution containing iodine from a source other than that of the calibration standards is used to verify acceptable calibration and to evaluate the ongoing performance of the instrument. The CCV should be analyzed after calibration, at least every 10 samples, and after the last sample in the analysis. A CCV should be of the same matrix as the standards used for calibration. A CCV result is considered acceptable when the result is within 90–110% of theoretical.

J. Calculations

If a reconstitution was performed, use the following equation:

$$\{[(C \times V) \times D]/WRA\}/10 = S$$

where C = sample concentration (ng/mL, sample solution reading on the curve); V = volume (mL, final volume after digestion); D = dilution factor (if not applicable, enter 1); WRA = weight (g) of reconstitution aliquoted during *sample preparation* (**d**); and S = sample concentration of iodine (µg/100 g reconstituted "as fed" basis).

If a reconstitution was not performed, use the following equation:

$$\{[(C \times V) \times D]/W\}/10 = S$$

Table 2. Laboratory results

	NIST SRM 1849a		Infant formula RTF milk based-1		Infant formula powder soy based		Infant formula powder milk based		Infant formula RTF milk based-2		Child formula powder		Adult nutritional powder low fat	
	EKVJ578	VJKY373	TJMN542	XKIP216	MNGN284	EPXW887	ZNPI092	YKLP059	HYJU890	XJDD334	GLBW236	GEUH577	CBNJ010	SNPZ056
Replicate	1	2	1	2	1	2	1	2	1	2	1	2	1	2
Lab	Iodine results, mg/kg[a]						Iodine results, µg/100 g[b]							
A	1.19	1.17	5.32	4.92	11.9	12.9	18.1	17.6	5.18	5.02	3.35	3.26	6.70	6.76
B	1.25	1.24	5.43	5.45	12.9	12.7	19.7	19.7	5.21	5.62	3.48	3.35	7.29	7.34
C	1.10	1.10	4.95	4.33	10.7	10.2	15.5	15.9	4.37	4.61	2.90	3.14	6.34	6.00
D	1.17	1.16	5.12	4.83	11.7	12.4	17.1	19.5	4.87	5.21	3.22	3.51	6.95	6.86
E	1.29	1.30	6.18	6.15	116[c]	116[c]	172[c]	172[c]	6.17	6.15	34.2[c]	34.5[c]	67.5[c]	67.5[c]
F	1.25[c]	1.11[c]	5.20	4.83	11.4	11.4	17.9	17.6	5.16	4.84	3.30	3.32	6.44	6.52
G	1.32	1.32	5.48	5.37	13.5	13.7	20.9	20.7	5.46	5.65	3.69	3.77	7.59	7.64
H	1.27	1.28	5.83	5.79	113[c]	115[c]	170[c]	168[c]	5.84	5.79	33.5[c]	33.5[c]	69.0[c]	68.8[c]
I	1.27	1.28	6.14	6.07	12.5	12.7	18.6	18.8	6.23	6.13	3.84	4.01	7.62	7.59
J	1.33	1.31	5.54	4.92	12.9	13.4	19.2	19.9	4.17	4.64	3.41	3.58	7.14	7.20
K	1.28	1.27	6.14	5.81	13.0	12.9	17.7	19.5	6.06	6.29	3.57	3.61	7.22	7.20
L	1.22	1.20	5.95	5.29	11.8	11.8	18.0	18.0	5.26	5.99	3.32	3.25	6.79	6.37
M	1.25	1.27	5.87	5.61	13.3	13.1	18.7	18.0	5.89	5.80	3.97	3.57	7.71	7.41

[a] NIST SRM 1849a results presented as mg/kg.

[b] µg/100 g reconstituted final product.

[c] Statistical outliers, data not included for statistical analysis.

where C = sample concentration (ng/mL, where sample solution reads on the curve); V = volume (mL, final volume after digestion); D = dilution factor (if not applicable, enter 1); W = sample size (g); and S = sample concentration of iodine (µg/100 g).

Results and Discussion

Seven samples were analyzed by 13 independent laboratories. These laboratories were from industry, contract research organizations, and government institutions. Laboratories were located in North America, Europe, and Asia. The seven samples for the collaborative study were selected to represent varying levels of iodine in a variety of applicable matrixes. The matrixes included an SRM, two different lots of milk-based infant formula RTF, a child powder formula, an adult nutritional low-fat powder, soy-based infant formula powder, and milk-based infant formula powder. Table 1 presents the diversity of ICP-MS instrument makes and models used by collaborating laboratories to generate data for the study. This table also attests the versatility of the method by showing that either of two digestion options provides the same results.

Laboratories were asked to record any deviation from the method protocol and to provide comments in general about the method. Of the 13 laboratories, three did not provide any comments. A significant majority of the remaining 10 study participants comments were related to the QC/study check criteria included on the test sample data summary spreadsheet. One of the QC/study check questions asked of participants was whether the analysis was performed on the same day as digestion, and if not, what was the length of time between digestion and analysis. Many participants responded yes or within 24 h. The amount of time from digestion to analysis for the remainder of the laboratories typically ranged from 2 to 7 days. One laboratory stated a period of 17 to 50 days between digestion and analysis. Additional QC/study check questions asked of participants included:

(*1*) Did you perform the analysis in standard (STD) mode?

(*2*) Were all individual back-calculated calibration standard concentrations within 90–110% of theoretical?

(*3*) Was the signal of the lowest calibration standard ≥1.5 times the blank signal?

(*4*) Were all CCB results run before, during, and after samples within ≤30% of the lowest calibration standard's nominal concentration?

(*5*) Were all digest blank results ≤30% of the lowest calibration standard's nominal concentration (≤0.075 ng/mL)?

(*6*) Were all CCV results (before, during, and after samples) within 90–110% of standard's nominal concentration (9.00–11.0 ppb)?

(*7*) Were all RSD values for iodine and praseodymium ≤5%?

Very few comments were provided pointing out values that exceeded these criteria. All participants indicated the analysis was performed in the STD mode. When limits were breached, exceedance was not significant. In three instances, digest blank or CCB results were 31.2, 32.4, and 34.4% of the lowest calibration standard. There were three occurrences where the individual back-calculated lowest calibration standard concentration (0.250 ppb) exceeded the assigned acceptance range exhibiting recoveries of 81.1, 83.3, and 113% of theoretical. One laboratory commented that the RSD of one sample analysis exceeded the assigned ≤5% criteria. This same laboratory commented "The last CCV (at end of run) was 8.84 ppb (ideally no lower than 9.00 ng/mL)." Other deviations noted by two laboratories were minor. One laboratory used sealed 55 mL digestion vessels and then transferred the samples "...to a final volume of 50 mL in another container." This same laboratory also used 0.25 µm syringe filters instead of the recommended 1 µm syringe filters. One laboratory altered the calibration standard scheme. Instead of using the recommended

0.250, 0.500, 1.00, 10.0, 50.0, and 100 ppb calibration standard curve points, a 5.00 ppb was added and the 100 ppb was deleted resulting in 0.250, 0.500, 1.00, 5.00, 10.0, and 50.0 ppb points. One participant mentioned issues with RSDs and IS drift when the method had not been performed on their instrument for a period of time but commented that adequate conditioning resolved the issues. The Study Director thoroughly reviewed all deviations and was confident, based on an overall assessment of the QC check information provided and statistical analysis of the results, that no impact to the data was evident.

All of the laboratories' results are presented in Table 2. Table **2012.15A** shows the statistical evaluations for all the samples analyzed in this multilaboratory testing study. The RSD_r ranged from 0.77 to 4.78%, and the RSD_R ranged from 5.42 to 11.5%. The HorRat values for all results ranged from 0.35 to 1.31%. Repeatability and reproducibility for all seven samples were below the limits set forth in AOAC SMPR **2012.008** (4). All 13 laboratories' data were included for statistical analysis for both RTF samples. Outliers for the powdered reconstituted samples and NIST SRM 1849a were removed prior to performing statistical analysis based on Cochran's and Grubbs' outlier tests.

Upon completion of the collaborative study, comparison of data for the reconstituted powders revealed five laboratories' results (Laboratories C, E, H, I, and L) were approximately 9 to 10 times higher than the other eight laboratories' data. The other eight laboratories' data agreed with values obtained during the SLV. The consistent factor of 9 to 10 suggested a calculation error, which agreed with the reconstitution factor (e.g., 225 g ÷ 25 g = 9). After correspondence with the five laboratories whose data were in question, it was evident that a misunderstanding of the calculation requirements for the reconstituted powders had occurred. The five laboratories had calculated the reconstituted powdered sample results on a dry basis instead of on an "as fed" basis. Laboratories C, I, and L submitted recalculated results prior to the collaborative study report submission due date, allowing inclusion of their data in the results table. Laboratory H submitted acceptable data but only after the due date. Laboratory E did not submit recalculated data. Since laboratories E and H recalculated reconstituted powder data were not received in time to include in the report, their original data were reported.

Several comments to strengthen the method were provided during the SPIFAN ERP meeting in March 2015:

Clarify in the method that it is not applicable to samples containing FD&C Red Dye No. 3 (erythrosine).

Point out the possible need for increased instrument maintenance when using the method. Include precautions about the lens and/or lens stack possibly requiring additional maintenance and that analysis would benefit from thoroughly conditioning the instrument.

Clarify the use and/or preparation of second source standards for CCV standard solutions.

If acidic sample matrixes are typically analyzed on the ICP-MS instrument, perform a thorough cleaning of the entire sample introduction system and appropriate conditioning prior to analyzing basic matrixes.

Clarify the importance of adhering to the peristaltic pump tubing sizes recommended for introducing IS and carrier solutions.

If possible, maintain a dedicated set of cones and/or lens.

These suggestions have been accepted and incorporated into the method. Also incorporated were minor modifications taken from comments provided by several collaborators as well as incorporation of components requiring clarification as suggested by the Study Director.

The overall results demonstrated that the method is fit-for-purpose to determine iodine levels in infant formula and adult/pediatric nutritional formula, and the Study Director recommended that it be adopted Official Final Action.

Recommendations

It was the recommendation of the Study Director that the method is fit-for-purpose in determining total iodine in infant formula and adult/pediatric nutritional formula by ICP-MS and that it be adopted as an AOAC Final Action *Official Method.* The AOAC ERP evaluated the data presented in the final report for the collaborative study of AOAC First Action *Official Method* **2012.15** in March 2015 after which the method was recommended Final Action status. Subsequently, the Official Methods Board approved the method for Final Action in June 2015.

Acknowledgments

We are very grateful to all laboratories that participated in the study, especially to the following collaborators (listed alphabetically based on their institution name):

Angela Song and Fang Tian (Abbott Nutrition International ANRD, China)

Kathryn Stanley and Mark W. Collison (Archer Daniels Midland Co., Decatur, IL)

Grant Fulford and Pee Yang (Covance, Madison, WI)

Yirong Xu, Sook Yain Chen, and KienHeng Lee (Covance, Singapore)

Marvin Boyd Jr, and Cheryl D. Stephenson (Eurofins Central Analytical Laboratories, New Orleans, LA)

Josh Messerly (Eurofins Nutrition Analysis Center, Des Moines, IA)

Anders K. Svaneborg and Per K.B. Nilsson (Eurofins Steins Laboratorium A-S, Denmark)

Sudhakar Yadlapalli (First Source Laboratory Solutions LLP, Hyderabad, India)

Cai Weihong [Guangzhou Quality Supervision and Testing Institute (GZ GQT), China]

Raymond Yu (National Center of Supervision and Inspection on Food Stuff Product Quality, Shanghai, China)

Xiaojun Deng (Shanghai Exit and Entry Inspection and Quarantine Bureau, China)

Wu Bolong (Test Center of Chinese Academy of Inspection and Quarantine, China)

Qin Xu and Lei Bao (The Food and Agricultural Products Testing Agency of Shandong CIQ, China).

A debt of gratitude goes out to Meagan (Dallman) Knautz, Covance Laboratories, Inc., Madison, WI, for her significant contributions during method development and leading up to and including the SLV. A sincere thank you goes to Darryl Sullivan, Covance Laboratories, Inc., Madison, WI; Robert Rankin, Infant Nutrition Council of America, Washington, DC; and James Wehrmann, Covance Laboratories, Inc., Madison, WI, for their guidance throughout the entire process. Special thanks

go to Colleen Pricco, Covance Laboratories, Inc., Madison, WI, for her invaluable assistance with labeling and dispatching samples to participating laboratories. Special thanks also go to Rebecca Hall, Covance Laboratories, Inc., Madison, WI, for her much appreciated last minute assistance with statistical analysis and tabling of results.

References

(1) Sullivan, D., & Zywicki, R. (2012) *J. AOAC Int.* **95**, 195–202. http://dx.doi.org/10.5740/jaoacint.11-350
(2) AOAC *Official Method* **2012.15** (2012) *Official Methods of Analysis of AOAC INTERNATIONAL*, 19th Ed., AOAC INTERNATIONAL, Rockville, MD
(3) AOAC SPIFAN (February 2013) Single-Laboratory Validation for Iodine Analysis in Infant Formula and Adult Nutritionals, Covance Laboratories, Inc., Madison, WI
(4) AOAC SMPR 2012.008, *Iodine in Infant Formula and Adult/Pediatric Nutritional Formula* (2013) *J. AOAC Int.* **96**, 486. http://dx.doi.org/10.5740/jaoac.int.2011.008

AOAC AWARDS- **2016** METHOD OF THE YEAR (ERP Chair Nomination Form)

Submission Date	2016-04-25 16:11:33
Name	Darryl Sullivan
Panel Name	SPIFAN
E-mail	darryl.sullivan@covance.com
Date	04-25-2016
AOAC Method Number	2011.10
Method Name	Determination of Vitamin B12 in Infant, Adult, and Pediatric Formulas by HPLC-UV and Column Switching
Rationale (based on selection criteria)	This is one of the most successful methods in the history of AOAC. It is not only approved as an AOAC Final Action Method, but it has also been approved by ISO, IDF, and Codex - as a Type II dispute resolution method. The SLV and MLV studies were both done extremely well and the data are amazing. The method will have a huge impact on the infant formula community, because it replaces a 50 year old microbiological method.
AOAC Method Number	2011.20
Method Name	Analysis of Nucleotide 5'-Mono phosphates in Infant Formulas by HPLC-UV
Rationale (based on selection criteria)	This is one of the most successful methods in the history of AOAC. It is not only approved as an AOAC Final Action Method, but it has also been approved by ISO, IDF, and Codex - as a Type II dispute resolution method. The SLV and MLV studies were both done extremely well and the data are amazing. The method will have a huge impact on the infant formula community, because it provides the first official method for measuring nucleotides - an extremely important nutrient that is added to almost all infant formula.
AOAC Method Number	2012.15
Method Name	Determination of Iodine in Infant Formula and Adult / Pediatric Nutritional Formula by Inductively Couple Plasma-Mass Spectrometry (ICP-MS)
Rationale (based on selection criteria)	This is one of the most successful methods in the history of AOAC. It is not only approved as an AOAC Final Action Method, but it has also been approved by ISO, IDF, and Codex - as a Type II dispute resolution method. The SLV and MLV studies were both done extremely well and the data are amazing. The method will have a huge impact on the infant formula community, because it replaces a 50 year ion specific electrode method that was prone to false positives and was extremely problematic. This is the first ICP-MS method to gain official method status for the determination of nutrients - and has set the framework for many more in the future.

INFANT FORMULA AND ADULT NUTRITIONALS

Pantothenic Acid (Vitamin B_5) in Infant Formula and Adult/Pediatric Nutritional Formula by Ultra-High Pressure Liquid Chromatography/Tandem Mass Spectrometry Method: Collaborative Study, Final Action 2012.16

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Collaborators: A. Aoude-Werner, S. Bandhari, G. Jaudzems, I. Malaviole, M. Nixon, T. Norloos, L.T. Tanderup, S. Tennyson, C. Tool, M. Torres, P. van der Burgh, M. Vermeulen, C. Weihong, B. Wu, L.K. Yap

In order to determine repeatability and reproducibility of AOAC First Action Method 2012.16 [Pantothenic Acid (Vitamin B_5) in Infant Formula and Adult/Pediatric Nutritional Formula by Ultra-High Pressure Liquid Chromatography/Tandem Mass Spectrometry], a collaborative study was organized. The study was divided in two parts: method setup and qualification of participants (part 1) and collaborative study participation (part 2). For part 1, each participating laboratory was asked to analyze two practice samples using the aforementioned method. Laboratories that provided results within a range of expected levels were qualified for part 2, during which each laboratory received 10 samples in blind duplicates. Results have been compared to the *Standard Method Performance Requirement* (SMPR®) 2012.009 established for pantothenic acid. Precision results (repeatability and reproducibility) were within the limits stated in the SMPR. Repeatability ranged from 1.3 to 3.3%, and reproducibility ranged from 4.1 to 7.0%. Horwitz ratio (HorRat) values were all <1, ranging from 0.33 to 0.69. The AOAC Expert Review Panel on Stakeholder Panel on Infant Formula and Adult Nutritionals Nutrient Methods determined that the data presented met the SMPR and recommended the method for Final Action status, which was then granted by the AOAC Official Methods Board.

Pantothenic acid (PA; vitamin B_5) is commonly present in foods of either plant or animal origin. This compound is an essential nutrient for humans, i.e., it is necessary to synthetize coenzyme-A, which is needed in a vast range of biological roles, such as metabolism of fatty acids; it also plays a key part in the Krebs cycle. Historically, determination of PA was performed with a microbiological assay using *Lactobacillus plantarum* and its turbidimetric growth (1, 2). Even if this method is rather sensitive, its specificity is limited when dealing with complex food matrixes.

More specific techniques have been tested for the analysis of this compound, such as indirect ELISA (3–5) and radioimmunoassay (6). Different LC methods have been developed as well, but the absence of a strong UV chromophore results in few methods using this detection available for the analyst. This detection difficulty can be circumvented by using highly selective MS. Andrieux et al. (7) used this approach in 2012, with a method combining a rapid sample preparation prior to the analysis of PA by ultra-high-pressure liquid chromatography (UHPLC) with a triple quadrupole MS detection. This method was proposed to the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) and was approved as First Action AOAC **2012.16** method (8), with a recommendation to advance to a multilaboratory collaborative study. This paper presents the results of that collaborative study.

Method

AOAC First Action Method **2012.16** was used, with minor modifications, mainly editorial.

AOAC Official Method 2012.16
Pantothenic Acid (Vitamin B_5) in Infant Formula and Adult/Pediatric Nutritional Formula
Ultra-High Pressure LC/MS/MS Method
First Acction 2012
Final Action 2015

ISO–AOAC Method

(Applicable to the determination of free PA in infant formula and adult/pediatric nutritional formula.)

Caution: Consult Material Safety Data Sheets prior to using chemicals and adhere to the safety precautions provided. Wear personal protective equipment when necessary.

Received May 19, 2015. Accepted by SG June 13, 2015.
[1] Corresponding author's e-mail: esther.campos-gimenez@rdls.nestle.com
The method was approved by the AOAC *Official Methods Board* as Final Action. *See* "Standards News," (2014) *Inside Laboratory Management*, July/August issue.

The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) invites method users to provide feedback on the Final Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author.

DOI: 10.5740/jaoacint.15-127

A. Principle

Extraction of PA using a 0.4 M ammonium acetate buffer solution. After filtration, the final solution is subjected to ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS).

B. Apparatus

(a) *Balances*.—With readability of 0.1 mg, capacity 210 g (AG204; Mettler-Toledo, Greifensee, Switzerland); with readability of 0.1 g, capacity 4100 g (PM4800 DeltaRange, Mettler-Toledo) or equivalent.

(b) *pH meter*.—Model 691 (Metrohm, Herisau, Switzerland), with readability of 0.01 pH unit or equivalent.

(c) *Homogenizer*.—Polytron PT3000 (drive unit), Aggregate PT-DA 3012 (Kinematics, Lucerne, Switzerland) or equivalent.

(d) *Stir plate with magnetic stirrers*.

(e) *Filters*.—Syringe filters, 0.22 µm pore size, 33 mm id, Millex-GV PVDF (EMD Millipore Corp., Billerica, MA). Membrane disc filters, 0.45 µm pore size (EMD Millipore Corp.) or equivalent.

(f) *UHPLC/MS/MS system*.—Acquity UPLC coupled with triple quadrupole detector equipped with electrospray ionization (ESI) source and T3 column (1.8 µm, 100 × 2.1 mm id; Waters Corp., Milford, MA) or equivalent.

C. Chemicals and Solvents

(a) *Standards*.—(*1*) *Calcium D-pantothenate*.—Sigma (St. Louis, MO) or equivalent. (*2*) *Calcium pantothenate-[$^{13}C_6$, $^{15}N_2$]*.—IsoSciences (King of Prussia, PA) or equivalent.

(b) *Enzyme*.—α-Amylase, Sigma A3176, from porcine pancreas, about 25 U/mg or equivalent.

(c) *Solvents*.—(*1*) *Acetonitrile*.—LC grade (Honeywell, Muskegon, MI; LC015-1, or equivalent). (*2*) *Water*.—>18 MΩ.

(d) *Ammonium acetate*.—ACS grade, >98% (Fluka 9690, Sigma, or equivalent).

(e) *Acetic acid*.—ACS grade (Marcon Chemicals, Center Valley, PA; 3121-46, or equivalent).

(f) *Formic acid*.—ACS grade (Sigma 695076, or equivalent).

(g) *1% Formic acid in water*.—ACS grade (Honeywell; LC452-1, or equivalent).

D. Preparation of Standard Solutions

(a) *PA stock solution (250 µg/mL)*.—Weigh 54.5 mg calcium pantothenate into a 200 mL volumetric flask (take into account the moisture content given in the supplier's certificate, or dry it to constant weight before use) and dilute to volume with water. Store aliquots at –20°C for no longer than 1 month before use.

(b) *PA intermediate solution (10 µg/mL)*.—Transfer 1 mL PA stock solution into a 25 mL volumetric flask and dilute to volume with water. Prepare this solution the day of use.

(c) *Calcium pantothenate-[$^{13}C_6$, $^{15}N_2$] internal standard (IS) stock solution (20 µg/mL)*.—Weigh 5.0 mg calcium pantothenate-[$^{13}C_6$, $^{15}N_2$] into a 250 mL volumetric flask and dilute to volume with water. Store aliquots at –20°C for no longer than 2 months before use.

(d) *Preparation of 5-level standard curve*.—Transfer appropriate volumes of the PA intermediate solution (10 µg/mL) into 10 mL volumetric flasks to obtain five different concentrations of PA (0.08, 0.16, 0.32, 0.64, and 1.2 µg/mL); add 500 µL IS stock solution (20 µg/mL) and dilute to volume with water. Store aliquots of these solutions at –20°C for no longer than 1 month before use.

(e) *Ammonium acetate, 400 mmol/L, pH 3.8 (used for sample extraction)*.—Into a 500 mL beaker, add 30.8 ± 0.10 g ammonium acetate. Add about 300 mL water and stir to dissolve with a magnetic stirrer. Adjust to pH 3.8 ± 0.1, carefully adding glacial acetic acid (about 150 mL is needed). Transfer into a 1000 mL volumetric flask and make up to volume with water. This solution is stable for 1 month at 4°C.

E. Sample Preparation and Extraction

(a) *Preparation of food samples*.—Weigh a 25.0 g sample portion of homogeneous solid samples (i.e., powdered infant formula or nutritionals). Add 200.0 g water at 40°C before mixing until a homogeneous suspension is obtained. A homogenizer can be used when necessary.

Note: If the product contains starch, add 50 mg α-amylase to the aforementioned suspension and incubate for 15 min at 40°C to decrease viscosity and facilitate handling. Mix liquid samples well to ensure homogeneity and continue directly to extraction.

(b) *Extraction*.—Weigh a 15.0 g aliquot of homogenized sample suspension (corresponding to 1.67 g sample portion) or 20.0 g liquid sample into a 50 mL volumetric flask. Add 25 mL 0.4 M ammonium acetate solution, pH 3.8. Dilute to volume with water. Add a stir bar and stir for 10 min. Filter a 20 mL portion through folded paper (Whatman grade 597½; GE Healthcare Bio-Sciences, Pittsburgh, PA). Run chromatographic analysis.

F. Analysis

(a) *Chromatographic analysis*.—Transfer a 1.0 mL aliquot of the filtrate obtained in **E**(**b**) into a 15 mL polypropylene tube (e.g., Falcon tube; Fisher Scientific, Pittsburgh, PA) containing 500 µL IS stock solution. It is essential to use the same IS stock solution that has been used to prepare the 5-level standard curve. Dilute the solution to 10.0 ± 1.0 mL with water, cap, and mix. Filter through a 0.22 µm syringe filter. Inject into the UHPLC/MS/MS system.

(b) *UHPLC conditions*.—Injection volume, 2 µL; column temperature, 30°C; flow rate, 0.45 mL/min; mobile phase A, 0.1% (v/v) formic acid in water; and mobile phase B, acetonitrile.

Equilibrate the chromatographic system at an initial mobile phase composition of 92% mobile phase A and 8% mobile phase B. Run the gradient program 0 to 2.2 min ramp from 92 to 80% mobile phase A; 2.2 to 2.4 min ramp from 80 to 50% mobile phase A; 50% A hold from 2.4 to 4.0 min; back to the initial mobile phase composition at 4.1 min; and hold until 7.0 min. Direct the UHPLC flow into the MS detector only between 0 and 2 min to prevent source fouling as much as possible.

(c) *MS/MS conditions*.—Positive ESI; capillary voltage, 2.2 kV; cone, 25 V; extractor, 3.0 V; source temperature, 140°C; desolvation temperature, 350°C; cone gas flow, 40 L/h; and desolvation gas flow, 700 L/h.

Run in single-reaction monitoring mode. Monitor the transitions *m/z* 220.2 → 90.1 for PA, and *m/z* 224.2 → 94.1 for the isotope-labeled IS, between 0 and 2.1 min. Set collision energy at 14 V. The dwell time for each monitored transition is 0.1 s. The last two values are indicative and need to be checked and optimized for each instrument used.

(d) *Identification*.—MS detection in the single-reaction monitoring includes simultaneous detection of molecular ions corresponding to PA and labeled IS. The selected mass transitions are *m/z* 220.2 → 90.1 and *m/z* 224.2 → 94.1, respectively.

(e) *Quantitation*.—Calculate for each standard the peak area ratio between PA and IS. Establish a 5-point calibration curve (ranging from 0.16 to 2.4 ng on column) by plotting peak area ratio versus PA concentration. Calculate the linear regression. It is recommended to use a weighted regression curve (1/x). Calculate the slope (S) and the intercept (I). Calculate the PA concentration, w, in (mg/100 g) using the following equation:

$$w = \frac{(A - I) \times V_1 \times V_3 \times 100}{S \times m \times V_2 \times 1000}$$

where A = peak area ratio PA/IS in the test solution; I = intercept of the calibration curve; S = slope of the calibration curve; V_1 = volume of the of sample extract, in mL (= 50); V_2 = volume of the filtrate pipetted, in mL (= 1); V_3 = final volume of the test solution, in mL (= 10 ± 1); m = mass of the test portion, in g; 100 = conversion to 100 g basis; and 1000 = conversion from µg to mg.

Collaborative Study

Part 1

Participanting laboratories received two practice samples. Laboratories set up the method described in this paper. Participants were asked to analyze each of the two practice samples in duplicate (two extractions from each reconstituted sample). Any deviation, such as necessity to substitute reagents, columns, apparatus, or instruments, was to be recorded and reported. Reporting to the Study Director was done electronically using a template. Laboratories were asked to give all areas obtained (both PA and labeled PA) for the standard curve as well as for the samples. Concerning the standard curve, participants were given the choice to either use linear regression or a weighted linear regression (with 1/x as weight). This decision was to be mentioned in the informatics template. Furthermore, different masses used during sample preparation were to be reported. After review by the Study Director, results within a range of expected levels were used to identify the laboratories that had the capability to run the analysis successfully. The laboratories were thus qualified for the second part of the study.

Table 1. Results of practice samples for 14 laboratories

		≤5%	≤15%	
Requirements (SMPR 2012.009)	Mean, mg/100 g	RSD_r, %[a]	RSD_R, %[b]	HorRat values
Infant formula powder, milk-based	4.48	2.1	5.3	0.59
Infant formula powder, soy-based	5.16	2.5	6.0	0.68

[a] RSD_r is the RSD of repeatability.
[b] RSD_R is the RSD of intermediate reproducibility.

Part 2

All qualified laboratories received a second shipment containing 10 products in blind duplicates (i.e., 20 samples) for the collaborative study. The products came from a set of infant formula and adult nutritional products (i.e., SPIFAN kit) aimed to represent the whole range of commercially available products. Laboratories were asked to analyze all the samples (single extraction from each reconstituted sample) on 2 days (10 samples/day). Each sample was assigned to either day 1 or day 2. Results were transmitted to the Study Director via a similar electronic template as the one used in part 1.

Statistical Evaluation

After data collection, outliers were detected using Cochran's and Grubbs' tests. Average PA concentrations, SDs of repeatability (S_r), and RSDs of repeatability (RSD_r) were estimated from the blind duplicates in the collaborative study samples. The duplicates were assigned to be analyzed on the same day. SDs of reproducibility (S_R), RSDs of reproducibility (RSD_R), and HorRat (Horwitz ratio) values (RSD_R/predicted RSD_R) were also estimated. Details on statistical analysis can be found in *Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis* of the *Official Methods of Analysis* of AOAC (9).

Table 2. Results of collaborative study samples for 14 laboratories

			≤5%	≤15%	
Requirements (SMPR 2012.009)	*n*[a]	Mean, mg/100 g	RSD_r, %	RSD_R, %	HorRat values
Adult nutritional RTF high-fat[b]	14	2.07	2.9	7.0	0.69
SRM 1849a	14	6.96	2.0	5.1	0.60
Child formula powder	14	5.91	2.8	4.9	0.57
Adult nutritional powder milk protein-based	13	2.59	1.9	5.0	0.51
Infant formula powder soy-based	13	5.04	2.8	4.7	0.53
Infant formula RTF milk-based	13	0.549	1.5	4.1	0.33
Adult nutritional powder low-fat	13	8.07	1.6	4.1	0.50
Adult nutritional RTF high protein	13	1.57	1.7	5.5	0.52
Infant elemental powder	14	6.65	3.3	5.4	0.63
Infant formula powder part hydrolyzed soy-based	14	3.85	1.3	5.3	0.57

[a] *n* = Number of laboratories (after removal of outliers).
[b] RTF = Ready-to-feed.

Table 3. Full set of data, part 1. All results are given in mg/100 g

Lab No.	Adult nutritional RTF high fat		SRM 1849a		Child formula powder		Adult nutritional powder milk protein based		Infant formula powder soy based	
1	2.05	2.09	7.01	6.80	5.61	5.84	2.51	2.46	5.14	5.22
2	2.00	1.91	6.66	6.78	5.93	6.03	2.64	2.63	4.86	4.82
3	2.26	2.18	7.66	7.34	6.22	6.12	2.80	2.78	5.56	5.48
4	1.71	1.80	7.01	6.93	6.00	5.84	2.66	2.64	4.82	5.23
5	1.96	1.89	6.90	6.83	5.88	5.66	2.49	2.48	4.67	4.97
6	1.98	2.07	6.91	7.08	5.75	5.91	2.64	2.66	5.15	5.08
7	1.99	2.00	6.82	6.70	5.77	5.84	2.53	2.61	4.85	4.90
8	2.14	2.15	7.11	7.43	6.64	6.17	2.68	2.87	5.35	5.21
9	2.13	1.99	6.56	6.52	5.68	5.62	2.52	2.48	4.83	4.93
10	2.13	2.08	6.36	6.77	5.78	5.96	2.55	2.60	4.74	5.15
11	2.18	2.17	6.82	6.79	5.76	5.74	2.44	2.39	4.85	4.98
12	2.22	2.34	6.66	6.71	5.56	5.82	2.59	2.51	4.91	4.83
13	2.02	2.13	7.81	7.61	6.26	6.77	2.77	2.85	6.23[a]	5.19[a]
14	2.16	2.27	7.12	7.09	6.01	6.18	2.34[a]	2.67[a]	5.20	5.26

[a] Outlier identified by the Cochran test.

Results and Discussion

Part 1

Sixteen laboratories initially agreed to participate in the collaborative study. One laboratory dropped out during part 1 due to issues related to availability of resources. One laboratory did not qualify for the second part of the collaborative study due to results out of the range of expected levels for both practice samples.

Results for the two practice samples can be found in Table 1. The nonqualified laboratory's results were not taken into account. Repeatability was 2.1 and 2.5%, respectively; reproducibility was 5.3 and 6.0%, respectively. HorRat values were below 1, at 0.59 and 0.68. As this precision estimate is calculated on nonblinded duplicates, it therefore cannot be used for the actual collaborative study. Several minor comments were addressed to the Study Director and were taken into account in the final version of the method, which is presented in this paper.

Part 2

Fourteen laboratories sent a complete set of results. The statistical evaluation can be found in Table 2. Precision results (repeatability and reproducibility) are well within the limits stated in the *Standard Method Performance Requirements* (SMPR®) 2012.009 (10). Repeatability ranged from 1.3 to 3.3%, and reproducibility ranged from 4.1 to 7.0%. HorRat values were all below 1, from 0.33 to 0.69.

Laboratory 14 was identified as an outlier for the product "Adult Nutritional Powder Milk Protein Based." Laboratory 13 was an outlier for the products "Infant Formula Powder Soy

Table 4. Full set of data, part 2. All results are given in mg/100 g

Lab No.	Infant formula RTF milk based		Adult nutritional powder low fat		Adult nutritional RTF high protein		Infant elemental powder		Infant formula powder part hydrolyzed soy based	
1	0.536	0.558	7.96	8.01	1.54	1.52	6.35	6.25	3.68	3.63
2	0.533	0.530	8.26	8.02	1.49	1.46	6.22	6.35	3.95	4.01
3	0.589	0.592	8.69	8.67	1.67	1.68	7.18	7.22	4.19	4.17
4	0.554	0.548	8.21	8.00	1.58	1.58	6.09	6.78	3.92	3.94
5	0.547	0.529	7.73	7.39	1.37	1.41	6.74	6.28	3.46	3.54
6	0.558	0.550	8.24	8.18	1.61	1.59	6.87	6.69	3.81	3.83
7	0.502	0.515	7.96	8.05	1.55	1.48	6.57	6.61	3.95	3.81
8	0.540	0.550	8.12	7.90	1.62	1.59	6.62	7.00	3.71	3.73
9	0.527	0.529	7.92	7.84	1.54	1.50	6.38	6.27	3.58	3.66
10	0.573	0.556	8.41	8.25	1.59	1.57	6.93	6.85	3.91	3.99
11	0.534	0.548	7.75	8.07	1.62	1.68	6.64	6.58	3.81	3.80
12	0.585	0.577	7.59	7.57	1.69	1.66	6.33	6.34	3.63	3.69
13	0.560[a]	0.670[a]	7.13[a]	8.55[a]	1.64[a]	1.45[a]	7.00	7.59	4.18	4.03
14	0.554	0.554	8.41	8.55	1.62	1.66	6.60	6.85	4.06	4.05

[a] Outlier identified by the Cochran test.

Based," "Infant Formula RTF Milk Based," Adult Nutritional Powder Low Fat," and "Adult Nutritional RTF High Protein." These results were therefore not taken into account for the statistical evaluation. The full set of data can be found in Tables 3 and 4.

Conclusions

Precision results obtained during this collaborative study show that method **2012.16** is fit for purpose for the analysis of PA in a wide selection of infant formula and child and adult nutrition products. Data were submitted to the AOAC Expert Review Panel (ERP) for review at the AOAC Mid-Year meeting held on March 18, 2015, in Gaithersburg, MD. The ERP determined that the data presented met the SMPR set by SPIFAN and hence recommended the method for Final Action. Final Action status was granted by the AOAC Official Methods Board.

Acknowledgments

We would like to specially thank Colleen Pricco at Covance Laboratories (Madison, WI) for dispatch of samples to participants. We also thank the following participants and their associates for participating in the study.

Melissa Nixon and Karen Schimpf, Abbott Laboratories (Columbus, OH)

Bolong Wu, Chinese Academy of Inspection and Quarantine (Beijing, China)

Marina Torres, Departamento de Desarrollo de Métodos LATU-Laboratorio Tecnológico del Uruguay (Montevideo, Uruguay)

Chelsey Tool and John Austad, Covance Laboratories (Madison, WI)

Lars Toftgaard Tanderup and Sabine Meng Jensen, Eurofins Steins Laboratorium A/S (Vejen, Denmark)

Greg Jaudzems, Nestlé Quality Assurance Center (Dublin, OH)

Ton Norloos, Nestlé Quality Assurance Center (Nunspeet, The Netherlands)

Patrice van der Burgh, FrieslandCampina LQS (Leeuwarden, The Netherlands)

Lai Kuan Yap and Soo Hui Chooi, Nestlé Quality Assurance Center (Shah Alam, Malaysia)

Sneh Bandhari, Silliker Inc. (Chicago Heights, IL)

Martijn Vermeulen, TNO Triskelion bv (Zeist, The Netherlands)

Isabelle Malaviole, Laboratoire AQUANAL (Pessac, France)

Dalal Aoude-Werner and Julie Dalmas-Le Grandois, Aérial (Illkirch, France)

Cai Weihong, Guangzhou Quality Supervision and Testing Institute (Guangzhou, China)

Steve Tennyson and Greg Hostetler, Perrigo Nutritionals (Georgia, VT)

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SPECIAL GUEST EDITOR SECTION

Determination of Triphenylmethane Dyes and Their Metabolites in Salmon, Catfish, and Shrimp by LC-MS/MS Using AOAC First Action Method 2012.25: Collaborative Study

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A collaborative study was conducted to evaluate the AOAC First Action 2012.25 LC-MS/MS analytical method for the determination of residues of three triphenylmethane dyes (malachite green, crystal violet, and brilliant green) and their metabolites (leucomalachite green and leucocrystal violet) in seafood. Fourteen laboratories from the United States, Canada, and the European Union member states participated in the study including national and state regulatory laboratories, university and national research laboratories, and private analytical testing laboratories. A variety of LC-MS/MS instruments were used for the analysis. Each participating laboratory received blinded test samples in duplicate of salmon, catfish, and shrimp consisting of negative control matrix; matrix fortified with residues at 0.42, 0.90, and 1.75 µg/kg; and samples of incurred matrix. The analytical results from each participating laboratory were evaluated for both quantitative residue determination and qualitative identification of targeted analytes. Results from statistical analysis showed that this method provided excellent trueness (generally ≥90% recovery) and precision (RSDr generally ≤10%, HorRat <1). The Study Directors recommend Method 2012.25 for Final Action status.

Guest edited as a special report on "Methods of Analysis for Residues and Chemical Contaminants in Aquaculture" by Joe Boison and Sherri Turnipseed.

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DOI: 10.5740/jaoacint.14-263

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Triphenylmethane dyes have been used as a treatment against parasite and fungus infections in aquacultured fish, beginning with the use of malachite green (MG) in the 1930s (1, 2). Structurally related triphenylmethane dyes such as crystal violet (CV) and brilliant green (BG) have similar therapeutic properties, and the leuco metabolites of such dyes can persist in edible fish muscle for months (3). Concerns regarding the toxicity and mutagenicity of the dyes and leuco metabolites have resulted in triphenylmethane dyes being prohibited for the treatment of fish to be used for human consumption by many countries including the United States and the European Union (EU) member states (4–6). Although international regulations ban the use of triphenylmethane dyes in aquaculture, the dyes are inexpensive and readily available.

Efficient monitoring of the food supply is needed to ensure these therapeutic dyes are not used in aquaculture, and numerous analytical methods have been published toward this goal. LC-MS/MS methods allow simultaneous determination of multiple triphenylmethane dye and leuco metabolite residues at and below a level of 1 µg/kg (7). One such LC-MS/MS method for determining three readily available triphenylmethane dyes (MG, CV, and BG) and the metabolites leucomalachite green (LMG) and leucocrystal violet (LCV) in seafood has recently been granted AOAC First Action Status (8). The method is based on a relatively simple acetonitrile extraction with hydroxylamine hydrochloride and magnesium sulfate. Residue quantification is normalized by the use of isotopically labeled internal standards and calibration based on extracted matrix standards. This paper describes the collaborative study of the AOAC First Action Method **2012.25** by 14 laboratories for the analysis of MG, CV, BG, LMG, and LCV residues in salmon, catfish, and shrimp.

Collaborative Study

Fourteen laboratories participated in this study. Laboratory participants were provided with intermediate standard solutions of the dyes, metabolites, and internal standards and homogenized salmon matrix for the purpose of method familiarization, as

well as the published AOAC First Action **2012.25** method (8). A detailed study protocol based on **2012.25** was also provided to the study participants for additional guidance. The collaborative study was designed to meet the requirements of a U.S. Food and Drug Administration (FDA) Foods Program level four full collaborative study chemical method validation (9).

Control filets of Channel catfish (*Ictalurus punctatus*) and Atlantic salmon (*Salmo salar*) were obtained from the FDA Center for Veterinary Medicine (CVM) Office of Research Aquaculture Program. Residue incurred catfish and salmon were produced at the CVM by exposing one catfish (2.2 kg) and one salmon (2.8 kg) to individual treatment baths at 25 and 12°C, respectively, containing a mixture of MG, CV, and BG each with a concentration of 2 µg/L. Each treated fish was placed in an exposure tank for 1 h, removed to a clean water tank, and then sacrificed 1 h after the exposure period. One control catfish and one control salmon were also collected from clean water tanks. To prepare homogenized portions of control and incurred muscle, skinless catfish filets (approximately 700 g) and salmon filets with attached skin (approximately 950 g) were separately ground with dry ice to produce fine powders. After overnight dry ice sublimation at –20°C, homogenized samples were sealed and stored at –80°C.

Frozen, peeled, and deveined white shrimp (*Litopenaeus vannemei*) were purchased commercially. This product was labeled as a product of Ecuador, farm-raised without the use of antibiotics or growth hormones. Approximately 900 g of these shrimp were ground with dry ice to produce a homogeneous control matrix as described above. Residue incurred shrimp was not available for this study. Surrogate incurred shrimp samples were produced by fortifying 2.00 g (±0.02 g) weighed portions of homogenized control shrimp with analytes to yield the following concentrations: 0.85 µg/kg MG, 0.75 µg/kg LMG, 1.18 µg/kg CV, 0.76 µg/kg LCV, and 1.50 µg/kg BG.

Study samples and standard solutions were shipped to each laboratory on dry ice, with instructions to store samples at –80°C and standards at –20°C until analysis. All analytical standards were obtained from Sigma-Aldrich (St. Louis, MO) including MG oxalate, LMG, CV chloride, LCV, BG (bisulfate salt), D5-MG picrate, D5-LMG, D6-CV trihydrate, and D6-LCV. All were indicated as Fluka analytical grade standards, except for LCV and BG that were available at the time only as reagent grade. Individual stock solutions of the dyes (MG, CV, and BG), leuco metabolites (LMG and LCV), and internal standards (MG-D5, LMG-D5, CV-D6, and LCV-D6) were prepared in acetonitrile with nominal concentration 100 µg/mL. Mixed standard solutions (1.000 µg/mL) were prepared in acetonitrile by combining the necessary volume of each stock solution to yield the exact final concentration for all the compounds. One mixed standard solution was prepared for the analytes (MG, LMG, CV, LCV, and BG) and one mixed standard solution for the internal standards (MG-D5, LMG-D5, CV-D6, and LCV-D6). Participants received vials of both mixed standard solutions along with instructions to prepare fresh working solutions from these standards on each day of analysis. Three sets of fish samples consisting of salmon, catfish, and shrimp matrix were also provided to each participating laboratory. Each set consisted of 17 tubes containing weighed portions (2.00 g ± 0.02 g) of homogenized matrix including: six tubes of negative control matrix labeled as calibrants, one tube of negative control matrix labeled as a QC, and 10 tubes of randomly numbered blinded test samples. Participants were instructed to fortify the six matrix calibrant samples prior to extraction with the appropriate amounts of working standard solutions to produce matrix fortified with 0, 0.25, 0.5, 1.0, 2.5, and 5.0 µg/kg of the analytes and 2.0 µg/kg of the internal standards. Participants were instructed to fortify the QC sample with analytes (1.0 µg/kg) and internal standards (2.0 µg/kg) after completion of the extraction procedure at the final extract reconstitution step. The QC sample was a post-extraction fortified calibrant that could be used to measure extraction losses when compared to the set of pre-extraction fortified calibrants. For further calibration comparisons in this collaborative study, participants were also requested to prepare and analyze a set of six solvent calibrants with concentrations to match the extracted matrix calibrants (0, 0.25, 0.5, 1.0, 2.5, and 5.0 µg/kg as tissue equivalents). Both the QC sample and solvent calibrant solutions were prepared in acetonitrile with an ascorbic acid concentration of 0.01% to match the composition of the extracted matrix calibrants. The 10 blinded test samples included two negative control matrix samples; six fortified samples with concentrations 0.42, 0.90, and 1.75 µg/kg (in duplicate); and two residue incurred samples (for salmon and catfish). For the shrimp matrix, for which residue incurred tissue was not available, two surrogate "incurred" samples were included in the set of blinded samples, each fortified with 0.75 µg/kg MG, 0.76 µg/kg LMG, 0.85 µg/kg CV, 1.18 µg/kg LCV, and 1.50 µg/kg BG. Study participants were instructed to fortify all 10 blinded samples with internal standard working solution (2.0 µg/kg) prior to extraction**.** Test samples for each of the three matrixes were labeled with unique letter and color coding (randomized between laboratories), which allowed these to be matched with the corresponding matrix calibrant tubes. The identity of the matrixes was not made known to the participants.

Both the **2012.25** method and the collaborative study protocol provided sufficient details to perform the LC-MS/MS analysis; however, participants were given flexibility to choose their analytical instrumentation and optimize the performance of their chosen system. Requirements given to the participants in order to select an appropriate triple quadrupole mass spectrometer were that the instrument would provide sensitivity to detect solvent solutions of the analytes with a concentration of 0.5 µg/L, and that two product ions would be collected for each analyte and one for each internal standard. In addition to the Waters Corp. (Milford, MA) LC-MS/MS system described in the **2012.25** method, participants were provided with optimized source parameters for an Agilent (Santa Clara, CA) 6490 LC-MS/MS system in the study protocol.

Finally, study participants were cautioned that triphenylmethane dyes and metabolites are light sensitive and require efforts to reduce background contamination. Participants were cautioned to reasonably protect samples and solutions from excessive light exposure (e.g., place in the dark or cover with foil when not in use), avoid black markers that are a known source of CV, and minimize instrument carryover by using an injection needle wash and/or injecting a blank water sample between blinded samples.

Each participating laboratory received a customized report spreadsheet matched to sample matrix and blinded sample coding that automatically displayed calibration curves and provided calculated concentrations as data was entered. On completion of the analyses, the participants returned this completed report sheet, as well as a summary of specific

instrument conditions used, any variations in the experimental procedure, raw data reports provided by the instrument, and chromatograms for all transitions monitored for each analyte and internal standard in all samples.

AOAC Official Method 2012.25
Residues of Three Triphenylmethane Dyes and Their Metabolites (Malachite Green, Leucomalachite Green, Crystal Violet, Leucocrystal Violet, and Brilliant Green) in Aquaculture Products
Liquid Chromatography/Tandem Mass Spectrometry
First Action 2012.25

[Applicable for the determination and confirmation of MG, LMG, CV, LCV, and BG in fish and shrimp muscle.]

Caution: Triphenylmethane dyes and leuco metabolites are toxic and known or suspected mutagens, carcinogens, and/or teratogens. Refer to Material Safety Data Sheets before handling any chemicals, wear safety glasses and appropriate personal protective equipment, and dispose of waste in an environmentally responsible manner in accordance with pertinent regulations.

A. Principle

Triphenylmethane dyes and their leuco metabolites, in the presence of hydroxylamine and anhydrous magnesium sulfate, are extracted from salmon, catfish, and shrimp tissue with acetonitrile. After evaporation of the extract, the residue is redissolved in acetonitrile/ascorbic acid and then analyzed using LC-MS/MS. Quantitative analysis uses extracted matrix calibrants and four isotopically labeled internal standards to correct for matrix effects and extraction losses.

B. Apparatus

(**a**) *Vortex mixer*

(**b**) *Rotary stirrer*.—Set to 100 rpm or multitube vortexer (platform shaker) set to 2500 rpm, or equivalent.

(**c**) *Centrifuge and tubes*.—Capable of accelerating 50 mL polypropylene centrifuge tubes (or equivalent) to 2000 × *g* and refrigerated to 4°C.

(**d**) *Transfer pipets*.—Disposable.

(**e**) *Nitrogen evaporator*.—Capable of heating sample tubes to 50°C.

(**f**) *Evaporation tubes*.—10–15 mL polypropylene tubes, or equivalent.

(**g**) *Microcentrifuge and tubes*.—Capable of accelerating microcentrifuge tubes containing 800 µL of volume to 20 000 × *g*.

(**h**) *PVDF syringe filters and syringes*.—0.45 µm, 13 mm Millex-HV (EMD Millipore Corp., Billerica, MA) and 1 mL disposable syringes, or equivalent.

(**i**) *Autosampler vials*.—Glass or polypropylene, with caps. Amber colored vials recommended to protect light sensitive compounds.

(**k**) *LC-MS/MS system*.—HPLC system equipped with pump, solvent degasser, autosampler, and column oven (Waters Corp. 2695, Agilent 1200 series, or equivalent). Triple quadrupole mass spectrometer system equipped with an electrospray ionization source for operation in the positive ion mode and capable of selected reaction monitoring (SRM) with at least two transitions/analyte and one transition/internal standard (Waters Corp. Quattro LCZ, Agilent 6490, or equivalent).

(**l**) *LC column*.—C_{18} stationary phase (100 × 2.1 mm, 3.5 µm) with C_{18} guard column (10 × 2.1 mm; Waters Corp. Symmetry), or equivalent.

C. Reagents

Note: All reagents should be, analytical HPLC or LC-MS grade

(**a**) *Acetonitrile*.

(**b**) *Hydroxylamine hydrochloride.*

(**c**) *Magnesium sulfate, anhydrous.*

(**d**) *Ammonium formate.*

(**e**) *Ascorbic acid.*

(**f**) *Formic acid.*

(**g**) *Water*.—Deionized, distilled.

(**h**) *MG oxalate*.—CAS No. 2437-29-8.

(**i**) *LMG*.—CAS No. 129-73-7.

(**j**) *CV chloride*.—CAS No. 548-62-9.

(**k**) *LCV*.—CAS No. 603-48-5.

(**l**) *BG*.—CAS No. 633-03-4.

(**m**) *D5-MG picrate*.—CAS No. 1258668-21-1.

(**n**) *D5-LMG*.—CAS No. 947601-82-3.

(**o**) *D6-CV trihydrate.*

(**p**) *D6-LCV*.—CAS No. 1173023-92-1.

D. Preparation of Reagent Solutions

(**a**) *Hydroxylamine solution in water (9.5 g hydroxylamine/L)*.—Dissolve 5.0 g hydroxylamine hydrochloride in deionized water and dilute to a final volume of 250 mL.

(**b**) *Ascorbic acid solution in water (1 g/L)*.—Dissolve 100 mg ascorbic acid in deionized water and dilute to a final volume of 100 mL.

(**c**) *Reconstitution solution*.—Combine 1 mL ascorbic acid solution (1 g/L) with 100 mL acetonitrile and mix.

(**d**) *Formic acid solution in water (5%, v/v)*.—Add 5 mL concentrated formic acid to approximately 90 mL deionized water and dilute with deionized water to a final volume of 100 mL.

(**e**) *Ammonium formate buffer (0.05 M, pH 4.5)*.—Dissolve 3.15 g ammonium formate in approximately 900 mL deionized water. Then add 5 mL formic acid solution (5%, v/v) and dilute with deionized water to a final volume of 1000 mL.

E. Preparation of Standard Solutions

(**a**) *Stock standard solutions*.—Prepare individual stock solutions of each dye, metabolite, and internal standard compound at a concentration of 100 µg/mL in acetonitrile, taking into account the purity and presence of counterions. Store all solutions in glass at –20°C and protect from light (stated stability = 1 year).

(**b**) *Mixed intermediate standard solutions*.—Prepare mixed intermediate standard solutions (1.000 µg/mL each compound) for the analytes (containing MG, LMG, CV, LCV, and BG) and the internal standards (containing MG-D5, LMG-D5, CV-D6,

and LCV-D6). To prepare each of these solutions, combine 1 mL of each individual stock solution required for the mixture and dilute to 100 mL final volume with acetonitrile. These solutions are stored in glass at –20°C and protected from light (stated stability = 1 month).

(**c**) *Working standard solutions*.—Prepare five to six working standard solutions. Six will be described here (WS 1–6). These are prepared by diluting aliquots (0, 50, 100, 200, 500, and 1000 µL, respectively) of the mixed intermediate standard solution of analytes to a final volume of 10 mL with acetonitrile. The resultant working standard solutions thus contain 0, 5, 10, 20, 50, and 100 µg/L, respectively, of the analytes. Prepare a working standard solution of internal standards by taking 400 µL of the mixed intermediate internal standard solution and diluting to a final volume of 10 mL with acetonitrile (final concentration 40 µg/L). Prepare all working standard solutions daily. These solutions may be kept at room temperature but protected from light.

F. Sample Preparation

(**a**) *Homogenization of samples*.—Homogenize muscle tissue with dry ice in a food processor to produce a finely ground powder. Allow the dry ice to sublime at –20°C and then store the homogenized tissues at –80°C. Homogenize salmon with attached skin, but for catfish, homogenize only the skinless filet. Remove shells, legs, and heads from shrimp prior to homogenization.

(**b**) *Extracted matrix calibrant samples*.—Accurately weigh 2.00 g (±0.02 g) portions of homogenized negative control tissue into each of six 50 mL disposable centrifuge tubes. Once thawed, fortify these samples (extracted calibrants 1–6) with 100 µL aliquots of WS1, WS2, WS3, WS4, WS5, and WS6, respectively. To each tube then add 100 µL internal standard working solution. The extracted matrix calibrant samples are thus fortified with 0, 0.25, 0.5, 1.0, 2.5, and 5.0 µg/kg of analytes and 2.0 µg/kg of internal standards. Allow calibrants to equilibrate 15 min protected from light before beginning the extraction with the addition of hydroxylamine solution. [*Note*: Method **2012.25** specifies that five extracted matrix calibrants are prepared in the range 0 to 2 µg/kg with concentrations 0, 0.5, 1.0, 1.5, and 2.0 µg/kg of analytes (8). For the collaborative study, the range was extended from 0 to 5 µg/kg to ensure that residues found in incurred samples would fall within the calibration range.]

(**c**) *Extraction of samples*.—Accurately weigh 2.00 g (±0.02 g) portions of homogenized tissue into 50 mL disposable centrifuge tubes and let thaw. Fortify thawed tissue with 100 µL internal standard working solution (2.0 µg/kg), and allow samples to equilibrate for 15 min while protected from light. Add hydroxylamine solution (9.5 g/L, 500 µL) to the samples, vortex mix briefly, and allow samples to stand in the dark for 10 min. Add acetonitrile (8 mL) and 1.0 g (±0.1) anhydrous magnesium sulfate to each tube. Vortex mix tubes (1 min, maximum speed), then shake tubes (10 min) using a rotary stirrer or a multitube vortexer. Centrifuge the tubes (2000 × *g*, 5 min, 4°C), and transfer all supernatant to a clean tube for evaporation. Evaporate the supernatant to dryness (50°C, N_2). For the salmon matrix, the point of dryness may be a viscous oil. Reconstitute the extracted matrix calibrant samples and test samples with 800 µL Reconstitution Solution. Vortex mix all samples sufficiently to break up dried extracts; for example, vortex mixing on high speed for 30 s followed by 10 min of mixing on a multitube vortexer ensures complete dissolution of analytes and internal standards. Transfer extracts to microcentrifuge tubes, centrifuge at 20 000 × *g* for 5 min, and filter (PVDF, 0.45 µm) into autosampler vials for LC-MS/MS analysis. The extraction results in a 2.5X concentration factor; therefore, a calibrant or sample fortified at 1.0 µg/kg in the seafood matrix will produce an extract with an equivalent concentration of 2.5 µg/L in the LC vial.

G. LC-MS/MS Analysis

(**a**) *LC*.—A Waters Corp. Symmetry C_{18} or comparable column is used, with or without a guard column. The mobile phase was made up of ammonium formate buffer (A, 0.05 M, pH 4.5) and acetonitrile (B). The gradient program is described in Table 1. The flow rate is 250 µL/min, the injection volume is 20 µL, and the column oven is set to 30°C. Potential carryover, particularly from CV can be reduced by injection of water between each test sample.

(**b**) *Triple quadrupole MS*.—A Waters Corp. Quattro LCZ triple quadrupole, or comparable instrument is used. The mass spectrometer is operated in the positive ion mode using electrospray ionization. Two SRM transitions are collected for each analyte and one SRM transition is collected for each internal standard; these transitions are detailed in Table 2, along with instrument parameters for the Waters Quattro LCZ system. Conditions are optimized so that all SRM transitions for the lowest concentration solvent calibrant are present with an acceptable S/N (≥3).

H. Screening

The method can be used to screen test samples against a single calibrant or to quantify samples using a full calibration curve. A screen is accomplished by extracting test samples along with a negative matrix control sample and a matrix sample fortified at 0.5 µg/kg. Concentration of the test sample is estimated by comparison of the quantification ion peak area ratio of sample:internal standard with the corresponding ratio for the fortified matrix sample. To confirm suspected positive samples, test samples should be extracted and analyzed in duplicate along with a range of fortified calibrants (including negative control). Test samples are then quantified using the calibration curve according to the quantification method described below in Section *I* (8).

Table 1. LC elution gradient

Time, min	A, % (ammonium formate buffer)	B, % (acetonitrile)
0	60	40
1	10	90
15	10	90
16	60	40
20	60	40

Table 2. MS/MS parameters for the Waters Corp. Quattro LCZ system

	SRM, *m/z*	Collision energy, eV	Cone voltage, V	Retention time, min
MG	329 → 313[a]	35	43	5.1
	329 → 208	35	43	5.1
MG-D5	334 → 318	40	30	5.1
CV	372 → 356[a,b]	40	25	5.6
	372 → 251[b]	35	25	5.6
CV-D6	378 → 362	40	25	5.6
BG	385 → 341[a]	35	35	6.0
	385 → 297	50	35	6.0
LMG	331 → 239[a]	25	25	7.8
	331 → 316	20	25	7.8
LMG-D5	336 → 239	25	25	7.8
LCV	374 → 358[a]	30	25	7.9
	374 → 239	25	25	7.9
LCV-D6	380 → 364	35	25	7.9
LBG	387 → 342[a]	30	25	10.9
	387 → 281	30	25	10.9

[a] Product ion transition used for quantification.

[b] An additional transition (*m/z* 372 → 340) was used by five laboratories for either quantification or for identification.

I. Quantification

(**a**) *Internal standards*.—MG-D5 is used as the internal standard for both MG and BG. All other analytes have their corresponding isotopically labeled internal standards (LMG-D5, CV-D6, and LCV-D6) incorporated into the method.

(**b**) *Calibration curves*.—For a given analyte, the quantification ion peak area ratios for analyte:corresponding internal standard (y-axis) are plotted versus concentration (x-axis) for the matrix calibrant samples. The resultant linear relationship ($R^2 \geq 0.95$) is used to calculate the concentration of the analyte in test samples using the equation y = mx + b, where m is the slope and b is the y intercept of the calibration curve**.**

J. Identification

Acceptable identification of an analyte can be determined according to either EU (10) or FDA (11) criteria. An analyte is considered to be present in a sample when:

(**a**) Its chromatographic retention time is ±2.5% (EU) or ±5% (FDA) of the average retention time for the corresponding non-zero matrix calibrant samples

(**b**) Its peak area ratio of qualitative ion:quantification ion is within the acceptable range of the corresponding average ratio for the non-zero extracted matrix calibrant samples. For the EU, this range is dependent on the peak ion ratio, ranging from ±20 to ±50 relative % (10). For the FDA, the acceptable range is ±10% absolute (11).

(**c**) The S/N must be ≥3 for both SRM transitions.

Results and Discussion

Method Performance

AOAC First Action Method **2012.25** proved to be fairly straightforward for participants, and all were able to complete the study and submit the required data. Participants were requested to perform their three sets of extractions and analyses within 3 weeks. Two laboratories completed their analyses in the first week from sample receipt, and the majority of laboratories completed or initiated their sample analysis within the second week. One laboratory completed the analyses in the fifth week. Performance of the method was evaluated based on the results from all 14 laboratories with regard to quantification and identification of each of the five analytes. Overall, the results of the study were excellent, with trueness generally ≥90% and RSDr generally ≤10%, with HorRat <1.

Ruggedness

A few deviations from, and variations within, the study protocol were noted by study participants and served to illustrate the ruggedness of the method. Deviations included differences in standard and sample storage temperatures, varying speeds of centrifugation, and differences in the pore size and material used for the final extract filtration. One laboratory stored standard solutions at –6°C, and three laboratories stored tissue samples at –20, –50, or –70°C, instead of the recommended –20°C for standards and –80°C for tissue samples. Six laboratories did not centrifuge samples at 2000 × *g* and eight laboratories did not microcentrifuge at 20 000 × *g*, but instead used a range of speeds (700 to 6000 × *g* for centrifuge and 10 000 to 30 000 × *g* for microcentrifuge), which were likely a function of available laboratory equipment. Final filtration was generally completed with PVDF syringe filters with 0.45 µm pore size as indicated in Method **2012.25**. Three laboratories reported that PVDF filters with 0.22 µm pore size were used, and one laboratory reported that 0.45 µm PTFE filter vials were used. None of the variations for storage temperature, centrifuge speed, or filtration appeared to have influenced method performance.

A variety of liquid chromatographic systems, including three ultra-HPLC (UHPLC) systems, were used in this study. Method **2012.25** and the study protocol provided for HPLC conditions, however, participants were given flexibility to design their own chromatographic separation to ensure that all analytes were retained sufficiently on their column. Variations were observed for injection volume, mobile phase gradient, flow rate, and column temperature. The primary concerns voiced by participants during the method familiarization phase involved the high percentage of acetonitrile (approximately 99% by volume) in reconstituted samples compared to the initial mobile phase composition of 40% acetonitrile. Participants were encouraged to adjust the gradient used and/or, given their sensitive instrumentation, decrease the injection volume in order to ensure analytes were suitably retained on the chromatographic column and detected. Five laboratories slowed down the initial (0–1 min) 40 to 90% acetonitrile gradient in Method **2012.25** (Table 1) by holding the initial acetonitrile composition at 10 or 20% for 0.5 to 3 min, then ramping up to 90% acetonitrile over 2 to 12 min. One laboratory used the mobile phase gradient described in Method **2012.25** gradient until 6 min, then dropped

Table 3. Statistical summary of interlaboratory study data

Analyte	Matrix	Analyte added, µg/kg	Statistical outlier lab No.[a]	No of labs	No. of replicates[b]	Trueness (recovery, %)	Mean concn, µg/kg	s_r	s_R	RSD_r, %	RSD_R, %	HorRat
MG	Salmon	0.42	10[c],2[d]	12	24	92.8	0.39	0.02	0.05	5.53	13.76	0.26
		0.90	None	14	28	92.2	0.83	0.05	0.13	6.13	16.22	0.35
		1.75	None	14	28	89.7	1.57	0.06	0.16	3.84	10.33	0.24
		Incurred	None	14	28		1.48	0.10	0.36	6.46	24.06	0.56
	Catfish	0.42	12[d],3[e],11[e]	11	22	90.5	0.38	0.04	0.05	10.64	14.15	0.27
		0.90	7[c],12[d]	12	24	77.8	0.70	0.04	0.23	5.31	32.61	0.68
		1.75	12[d]	13	25	79.4	1.39	0.09	0.36	6.79	25.87	0.60
		Incurred	None	14	28		3.23	0.18	1.18	5.47	36.38	0.96
	Shrimp	0.42	None	13	26	100.0	0.42	0.04	0.14	9.82	32.31	0.63
		0.90	None	13	26	97.8	0.88	0.08	0.22	9.37	25.55	0.55
		1.75	4[c]	12	24	88.0	1.54	0.09	0.56	6.04	36.06	0.85
		Inc:0.75	None	13	26	94.7	0.71	0.05	0.16	6.98	22.54	0.47
LMG	Salmon	0.42	2[d],10[e],14[e]	11	22	97.6	0.41	0.02	0.03	3.94	6.72	0.13
		0.90	10[d]	13	26	103.3	0.93	0.03	0.06	3.11	6.59	0.14
		1.75	None	14	28	97.1	1.70	0.09	0.19	5.14	11.17	0.27
		Incurred	None	14	28		0.81	0.03	0.12	3.57	14.25	0.31
	Catfish	0.42	12[d]	13	26	104.8	0.44	0.01	0.02	2.89	5.45	0.11
		0.90	12[d], 5[e], 6[e]	11	22	107.8	0.97	0.03	0.03	2.84	3.50	0.08
		1.75	12[d]	13	25	101.1	1.77	0.08	0.10	4.71	5.91	0.14
		Incurred	12[c]	13	26		2.39	0.05	0.16	2.20	6.81	0.17
	Shrimp	0.42	11[c]	13	26	102.4	0.43	0.01	0.04	3.22	8.98	0.17
		0.90	None	14	28	106.7	0.96	0.04	0.07	3.69	7.07	0.16
		1.75	None	14	28	101.7	1.78	0.07	0.16	4.14	8.81	0.21
		Inc:0.76	4[e]	13	26	99.8	0.76	0.03	0.05	3.64	6.22	0.13
CV	Salmon	0.42	2[d], 13[d]	12	24	102.4	0.43	0.02	0.03	4.22	6.15	0.12
		0.90	11[c]	13	26	103.3	0.93	0.05	0.11	5.49	11.38	0.25
		1.75	11[c]	13	26	99.4	1.74	0.05	0.18	3.06	10.36	0.25
		Incurred	11[c]	13	26		0.03	0.01	0.05	42.14	159.6	2.10
	Catfish	0.42	14[c]	12	24	102.4	0.43	0.02	0.05	5.15	11.04	0.21
		0.90	None	13	26	103.3	0.93	0.04	0.11	4.20	12.28	0.27
		1.75	14[c]	12	23	98.3	1.72	0.03	0.17	3.66	10.10	0.24
		Incurred	14[c], 6[c]	11	22		0.15	0.04	0.05	28.64	31.77	0.53
	Shrimp	0.42	None	14	28	102.4	0.43	0.06	0.09	13.21	21.41	0.42
		0.90	14[d]	13	26	104.9	0.94	0.03	0.10	3.11	10.70	0.23
		1.75	14[c]	13	26	99.4	1.74	0.05	0.23	3.10	13.09	0.31
		Inc:0.85	2[d], 8[c]	12	24	97.6	0.83	0.02	0.07	2.30	8.70	0.19
LCV	Salmon	0.42	2[d]	13	26	97.6	0.41	0.02	0.05	5.09	11.26	0.22
		0.90	None	14	28	102.2	0.92	0.05	0.09	5.50	9.48	0.21
		1.75	11[c], 3[c]	12	24	102.8	1.80	0.04	0.15	2.19	8.10	0.20
		Incurred	None	14	28		0.39	0.02	0.06	6.17	14.63	0.28
	Catfish	0.42	12[d]	13	26	109.5	0.46	0.02	0.05	4.60	10.34	0.20
		0.90	12[d]	13	26	107.8	0.97	0.03	0.08	3.38	8.04	0.18
		1.75	4[c], 12[d]	12	23	103.4	1.81	0.06	0.11	3.19	6.30	0.15
		Incurred	12[c]	13	26		4.04	0.16	0.40	4.01	9.98	0.27

Table 3. (*continued*)

Analyte	Matrix	Analyte added, µg/kg	Statistical outlier lab No.[a]	No of labs	No. of replicates[b]	Trueness (recovery, %)	Mean concn, µg/kg	s_r	s_R	RSD_r, %	RSD_R, %	HorRat
	Shrimp	0.42	None	14	28	104.8	0.44	0.03	0.03	6.48	7.81	0.15
		0.90	None	14	28	106.7	0.96	0.04	0.06	3.83	6.11	0.13
		1.75	None	14	28	102.3	1.79	0.08	0.12	4.36	6.52	0.16
		Inc:1.18	None	14	28	98.3	1.16	0.04	0.06	3.39	5.40	0.12
BG	Salmon	0.42	10[c], 2[d]	12	24	104.8	0.44	0.04	0.08	8.02	19.07	0.37
		0.90	None	14	28	101.1	0.91	0.07	0.15	7.55	16.96	0.37
		1.75	None	14	28	96.0	1.68	0.09	0.22	5.31	12.98	0.31
		Incurred	None	14	28		1.48	0.12	0.31	8.21	20.75	0.49
	Catfish	0.42	12[c], 11[e]	12	24	104.8	0.44	0.02	0.09	5.17	21.51	0.42
		0.90	None	14	28	101.1	0.91	0.05	0.36	5.90	39.36	0.86
		1.75	None	14	27	97.7	1.71	0.14	0.59	8.23	34.46	0.83
		Incurred	2[e]	13	26		1.07	0.05	0.25	4.98	23.66	0.53
	Shrimp	0.42	None	13	26	107.1	0.45	0.04	0.14	9.57	30.92	0.61
		0.90	None	13	26	107.8	0.97	0.10	0.28	10.13	28.90	0.64
		1.75	None	13	26	99.4	1.74	0.18	0.70	10.57	40.48	0.97
		Inc:1.5	None	13	26	102.7	1.54	0.07	0.42	4.31	27.57	0.65

[a] Laboratory numbers were randomly assigned and do not correspond to the sequence listed on either the title page or in the *Acknowledgments*.

[b] No. of replicates after removal of invalid data and outliers.

[c] Outlier by the Cochran test.

[d] Outlier by Grubbs 1 test.

[e] Outlier by Grubbs 2 test.

the composition of acetonitrile from 90 to 40% from 6–7 min and held at 40% for a total run time of 12 min. Compared to Method **2012.25** (20 min run time), overall chromatographic run times for the six laboratories that adjusted the mobile phase ranged from 7 to 25 min. Many participants made adjustments to the injection volume, with eight laboratories reducing the injection volume from 20 to 2, 3, 5, or 10 µL. Two laboratories reduced the column flow rate from 250 to 200 µL/min, one increased flow rate to 300 µL/min, and three laboratories used column temperatures of 25 or 35°C instead of the method stated 30°C. Twelve participants used the exact column specified in the protocol, and the remaining laboratories used a Phenomenex (Torrance, CA) Prodigy ODS-3 100 × 2 mm, 3 µm or a Waters Corp. Atlantis dC_{18} 100×2.1 mm, 3.5 µm column. Most participants used a guard column, although four did not.

A variety of triple quadrupole mass spectrometers were used by the participating laboratories, including AB Sciex (Framingham, MA) 5500 QTrap (5), AB Sciex 4000 QTrap (2), AB Sciex 3000, Agilent 6490 (2), Agilent 6460, Thermo Scientific (San Jose, CA) TSQ Vantage, Thermo Scientific Quantum Discovery Max, Waters Corp. Micromass Quattro Micro API, Waters Corp. Acuity TQD, and Waters Corp. Quattro Premiere XE. All LC-MS/MS systems used in this study provided suitable data. Participants were requested to optimize mass spectrometer source and ionization parameters to yield acceptable response for the desired analyte range. Two participants analyzed extracts from each test sample on both Agilent and AB Sciex systems. These laboratories obtained comparable study results from their Agilent 6460/AB Sciex 4000 or Agilent 6490/AB Sciex 5500 QTrap analyses, further illustrating the suitability of this method for varied instrumentation. Mass spectrometric transitions provided in the method worked well for most analytes (Table 2). In the case of CV, some participants found that the qualitative transition (*m/z* 372→251) provided a peak with low signal. As a result, four participants used a substitute transition for qualitative purposes (*m/z* 372→340). One laboratory inadvertently used this alternate transition for quantification of the catfish and shrimp matrixes. Transition *m/z* 372→340, when used, provided acceptable results. Although MS/MS parameters and retention times were listed in the First Action method and in Table 2 for the metabolite leucobrilliant green (LBG), this analyte was not specifically included in Method **2012.25** LC-MS/MS validation nor required for inclusion in the collaborative study. Analytical standards of LBG are not commercially available and have limited stability (12, 13). Nevertheless, three of the participating laboratories submitted data for the LBG transitions. None of the study samples were fortified with LBG, but as the expected metabolite from BG exposure, LBG may be present in incurred samples. On review of the data from three laboratories, potential responses for the LBG transitions for incurred salmon and catfish were not large enough to be distinguished from nonincurred samples. Without an LBG standard, MS/MS optimization and retention time comparison could not be performed by these laboratories. The First Action method authors detected the LBG metabolite in the concentration range 8 to 18 µg/kg for trout placed in a 100 µg/L

Table 4(a). CC_α and CC_β for triphenylmethane dyes and metabolites in seafood matrix

	CCα, µg/kg					CC_β, µg/kg				
	MG	LMG	CV	LCV	BG	MG	LMG	CV	LCV	BG
Salmon	0.24	0.17	0.18	0.29	0.35	0.27	0.19	0.20	0.33	0.38
Catfish	0.27	0.17	0.21	0.15	0.41	0.31	0.19	0.24	0.17	0.45
Shrimp	0.29	0.14	0.32	0.28	0.43	0.33	0.16	0.36	0.32	0.49

Table 4(b). MDL and LOQ for triphenylmethane dyes and metabolites in seafood matrix

	MDL, µg/kg					LOQ, µg/kg				
Salmon	0.12	0.07	0.07	0.11	0.21	0.47	0.27	0.26	0.45	0.85
Catfish	0.13	0.06	0.12	0.12	0.30	0.53	0.24	0.47	0.47	1.22
Shrimp	0.33	0.09	0.23	0.09	0.34	1.35	0.38	0.91	0.35	1.35

BG treatment bath for 1 h (12). By comparison, the salmon and catfish incurred for this collaborative study were exposed to only 2 µg/L of BG for 1 h.

Preliminary Data Analysis

All reported data were compiled and examined for validity. Data were omitted for cause when participants reported specific difficulties, such as a sample being lost due to spillage, or inadvertent combination with another sample. While Method **2012.25** allows the measure of linear correlation to be as low as $R^2 = 0.95$, in this collaborative study, single outlier calibration points were excluded from the calibration when R^2 was less than 0.99 and the omission of one point would result in $R^2 \geq 0.99$ correlation for the remaining five calibrants. In three instances (CV in catfish for one laboratory, MG and BG in shrimp for another), linear correlation could not be achieved by deletion of a single calibration point. For these three, all data reported by the laboratory for the particular analyte in the particular matrix were omitted from the statistical analysis.

Statistical Analysis

A determination of repeatability and reproducibility was performed using the AOAC International Interlaboratory Study Workbook for Blind (Unpaired) Replicates, v. 2.1 (14), which was developed to implement the AOAC INTERNATIONAL guidelines for the AOAC Official Method Program (15).

Table 5. Comparison of the accuracy (trueness and precision) of 0.9 µg/kg fortified samples using three different calibration methods for residue quantification; data represents duplicate matrix spikes from 10 laboratories (n = 20)

	MG		LMG		CV		LCV		BG	
	Trueness, (avg. recovery, %)	RSD, %	Trueness, (avg. recovery, %)	RSD, %	Trueness, (avg. recovery, %)	RSD, %	Trueness, (avg. recovery, %)	RSD, %	Trueness, (avg. recovery, %)	RSD, %
Salmon										
Extracted matrix[a]	95.3	17.7	101.1	11.9	99.5	8.7	102.4	8.8	99.1	14.8
Post-extraction fortified matrix[b]	96.8	19.2	112.4	11.3	113.4	8.4	116.5	11.0	162.8	28.0
Solvent[c]	84.6	14.6	101.2	18.5	104.1	10.5	103.2	10.8	128.4	40.3
Catfish										
Extracted matrix	77.5	24.2	107.7	6.3	101.8	8.6	105.2	6.7	90.4	26.2
Post-extraction fortified matrix	88.8	22.9	116.2	5.7	115.1	6.0	115.2	4.7	163.2	30.5
Solvent	79.3	25.9	104.2	7.4	113.1	10.8	95.5	18.8	140.1	42.6
Shrimp										
Extracted matrix	95.3	17.7	105.4	7.8	105.6	15.9	105.9	5.6	101.0	24.7
Post-extraction fortified matrix	105.5	15.3	118.2	7.2	125.7	21.0	113.3	7.0	145.2	19.5
Solvent	92.9	14.5	102.0	12.0	117.8	27.8	102.9	19.7	120.3	27.6

[a] Matrix fortified at calibration concentrations and then extracted to produce a set of six standards: 0, 0.25, 0.5, 1.0, 2.5, and 5.0 µg/kg (tissue equivalent[d]).

[b] Calibration standard based on one portion of tissue extracted and the matrix extract fortified at a concentrations of 1.0 µg/kg (tissue equivalent).

[c] Six standards prepared in solution at tissue equivalent concentrations.

[d] Concentrations are equivalent to the amount present in the 2 g sample portion. Method results in a 2.5-fold concentration of residues in the extracts.

Table 6. Residue identification summary

Analyte	Matrix	Concn of analytes, µg/kg	Analytes identified, %, *n* = 28		Concn (>0.05 µg/kg) of analytes in individual blank samples[c], µg/kg
			EU[a]	FDA[b]	
MG	Salmon	Blank	7	7	0.10, 0.11
		0.42	93	93	
		0.90	100	100	
		1.75	100	100	
		Incurred	100	100	
	Catfish	Blank	0	0	
		0.42	96	100	
		0.90	93	93	
		1.75	100	100	
		Incurred	93	100	
	Shrimp	Blank	4	4	
		0.42	100	100	
		0.90	100	100	
		1.75	93	93	
		Incurred	82	89	
LMG	Salmon	Blank	4	4	0.08
		0.42	100	100	
		0.90	100	100	
		1.75	100	100	
		Incurred	100	100	
	Catfish	Blank	11	11	
		0.42	100	100	
		0.90	100	100	
		1.75	100	100	
		Incurred	100	100	
	Shrimp	Blank	0	0	
		0.42	100	100	
		0.90	100	100	
		1.75	100	100	
		Incurred	100	100	
CV	Salmon	Blank	57	57	0.14
		0.42	100	100	
		0.90	100	100	
		1.75	100	100	
		Incurred	79	79	
	Catfish	Blank	76	76	
		0.42	100	100	
		0.90	100	100	
		1.75	100	100	
		Incurred	89	93	
	Shrimp	Blank	46	46	0.30
		0.42	100	100	
		0.90	100	96	
		1.75	100	100	
		Incurred	100	100	
LCV	Salmon	Blank	50	43	0.06, 0.08
		0.42	100	86	
		0.90	100	100	
		1.75	100	100	
		Incurred	100	96	
	Catfish	Blank	33	22	0.08, 0.12, 0.13
		0.42	100	100	
		0.90	96	96	
		1.75	100	100	
		Incurred	93	93	
	Shrimp	Blank	0	0	
		0.42	93	93	
		0.90	93	86	
		1.75	93	89	
		Incurred	96	89	
BG	Salmon	Blank	18	18	0.13
		0.42	93	89	
		0.90	96	96	
		1.75	100	100	
		Incurred	100	100	
	Catfish	Blank	15	15	0.08, 0.08
		0.42	100	100	
		0.90	100	100	
		1.75	100	100	
		Incurred	100	100	
	Shrimp	Blank	14	14	
		0.42	96	96	
		0.90	100	100	
		1.75	96	96	
		Incurred	100	100	

[a] ±20–50% relative, based on value.

[b] ±10% absolute.

[c] Only blank samples that meet confirmation criteria for the analyte/matrix pair and have a calculated concentration >0.05 µg/kg are reported.

Table 7. Analytical screening results for samples compared only to a single 0.5 µg/kg extracted matrix calibrant (n = 28[a]; 14 laboratories with duplicate samples at each concentration level)

	Samples with peak area response >0.5 µg/kg calibrant, %				
	MG	LMG	CV	LCV	BG
Salmon					
Negative control	0	0	0	0	0
Spike level 0.42 µg/kg	7	0	7	4	18
Spike level 0.90 µg/kg	100	100	100	100	100
Spike level 1.75 µg/kg	100	100	100	100	100
Incurred	100	100	0[b]	0[b]	100
Catfish					
Negative control	0	0	0	0	0
Spike level 0.42 µg/kg	14	11	5	32	25
Spike level 0.90 µg/kg	79	100	100	100	96
Spike level 1.75 µg/kg	100	100	100	100	100
Incurred	100	100	4[b]	100	100
Shrimp					
Negative control	0	0	0	0	0
Spike level 0.42 µg/kg	31	0	21	4	23
Spike level 0.90 µg/kg	96	100	100	100	100
Spike level 1.75 µg/kg	100	100	100	100	100
Incurred	96[c]	100	100	100	100

[a] Data excluded in the case of reported cause and for calibration curve nonlinearity; statistical outliers were not excluded.

[b] Mean concentrations of incurred samples were <0.4 µg/kg (0.03 for CV in salmon, 0.15 for CV in catfish, and 0.39 for LCV in salmon).

[c] Mean concentration found for MG incurred shrimp was 0.71 µg/kg.

Repeatability SDs (s_r), reproducibility SDs (s_R), repeatability RSDs (RSD_r), reproducibility RSDs (RSD_R), and number of statistical outliers are presented in Table 3. HorRat values are also presented in this table and are calculated as RSD_R (observed)/RSD_R (predicted), where the RSD_R (predicted) is calculated using the equation $RSD_R = 2C^{-0.1505}$, where C is the measured analyte concentration in decimal mass units. Cochran, Grubbs, and double Grubbs tests were used to remove statistical outliers where appropriate. When one data point was deemed to be an outlier, both replicates for that concentration level for that laboratory were excluded from the data set. The n = 14 number of participating laboratories permitted data from up to three laboratories to be excluded at each concentration level.

Analyte Quantification

Overall, the analytical results in all matrixes were excellent for the test samples fortified at 0.42, 0.90 and 1.75 µg/kg, as can be seen in Table 3. Trueness ranged from 88 to 108% recovery for analytes in all matrixes except for MG in catfish, which yielded lower recoveries (78–79%). RSD_r values were generally ≤10%, except in the case of low level incurred samples (CV in salmon and catfish). HorRat values were uniformly very low (<1). The sole exception was CV incurred salmon, with a HorRat of 2.1. The exposure of salmon to a low concentration mixture of the analytes for 1 h was not sufficient to provide a significant concentration of CV residue in the salmon muscle. The higher HorRat for CV incurred salmon is a clear indication that the mean measured concentration of 0.03 µg/kg of CV is below the LOQ for this method.

In the single-laboratory validation of the First Action method in trout matrix, the authors determined the decision limit (CC_α) and the detection capability (CC_β) for each analyte (12). CC_α ranged from 0.13 to 0.42 µg/kg for the five analytes in trout matrix, and CC_β ranged from 0.17 to 0.54 µg/kg. In trout matrix, the method was determined to have the greatest sensitivity for CV and the least for LCV. From collaborative study data, CC_α and CC_β were determined from the quantitative product ion transition[1] for each analyte in each matrix from the extracted calibration curves according to ISO 11843-2 (16) and Commission Decision 2002/657/EC (10). The medians of individual CC_α and CC_β values determined for each of the 14 laboratories are reported in Table 4a, as recommended in ISO 11843-2 for a multilaboratory validation (16). Individual values of CC_α were >1 µg/kg only for one salmon analysis by one laboratory, where calibration data for MG and BG yielded CC_α values of 1.23 and 1.16 µg/kg, respectively. For the overall median data, CC_α ranged from 0.14 to 0.42 µg/kg for the five analytes in salmon, catfish, and shrimp, and CC_β ranged from 0.16 to 0.47 µg/kg. Collaborative study results for salmon, catfish, and shrimp were consistent with those reported in the First Action method validation for trout (12).

In addition to CC_α and CC_β, the method detection level (MDL) and LOQ were calculated from the 0.42 µg/kg fortified sample data at the 99% confidence level (17). The MDL was calculated as the SD of the 0.42 µg/kg sample results (n = 28, 14 laboratories with duplicate samples) multiplied by the Students t-value at the 99 % confidence interval (one tailed) for that number of samples. The LOQ was determined as 10 times the SD of the 0.42 µg/kg sample results. Results for the MDL and LOQ for the different analytes and matrixes are summarized in Table 4b. Samples were excluded from the MDL and LOQ determinations if they had been identified as statistical outliers or excluded for cause, and the total number of samples (degrees of freedom) was adjusted accordingly. All MDLs were less than the lowest concentration level for the fortified samples, and the majority of the LOQs were determined to be below the 1.0 µg/kg level of concern. Considering these data are compared across 14 different laboratories using different analytical instrumentation, the low MDLs and LOQs highlight the sensitivity and robustness of this method. MDLs and LOQs calculated from validation data produced by a single laboratory would be expected to be significantly lower; however, for the collaborative study, each laboratory only generated results for two samples/matrix at the 0.42 µg/kg concentration, which

[1] For CC_α calculations, quantitative product ion transition data was calculated as the peak area ratio relative to the internal standard. At the zero calibration level, most participating laboratories reported numerical peak area data for small peaks or noise detected at the retention time of the analyte, while other labs reported the value "0". It was beyond the scope of the study to obtain non-zero noise measurements from each laboratory. Of the 210 individual CC_α calculations, 30% were based on calibration data sets that included the value of "0" for the peak area ratio at the zero calibration level.

would not produce statistically relevant determinations of MDL and LOQ.

The method accuracy generally yielded analyte trueness greater than 90%. In order to determine the extent of correction provided by the combination of extracted matrix calibrants and internal standard correction, participants were requested to concurrently analyze a set of solvent calibrants and a single post-extraction fortified matrix calibrant (QC, 1.0 µg/kg) along with the study samples. Internal standard corrected peak area ratios for the 0.90 ng/g fortified sample results generated by 10 laboratories were converted to concentrations using the three different methods of calibration: (*1*) extracted matrix calibration curves (the quantitative method used in this collaborative study), (*2*) single point calibration against the post-extraction fortified matrix QC calibrant, and (*3*) solvent calibration curve (Table 5). For the single point QC calibration method, the 0.90 µg/kg fortified samples were normalized relative to the 1.0 µg/kg QC calibrant.

There was generally good agreement among the three calibration methods for all analytes except for BG, where the post-extraction fortified QC matrix calibrant and solvent calibration curve overpredicted the concentration of the residue level and often led to poorer repeatability. Differences in method performance between BG and MG-D5 may account for variations in the analyte accuracy for BG. BG quantification was studied in greater detail with respect to internal standard correction and matrix effects in a complementary single-laboratory validation study of this method (18). While the post-extraction fortified QC matrix calibrant produced the highest calculated recoveries generated by the three methods, there was generally little difference between the average recoveries generated by the extracted matrix calibration curves and the solvent calibration curves for the analytes with matched isotopically-labeled internal standards (MG, LMG, CV, and LCV). The variance in analyte recovery using solvent calibration curves was also generally the largest. A more extensive investigation of this phenomenon has been reported (18). In that work, method accuracy determined from data collected by a single laboratory for MG, LMG, CV, and LCV was found to be generally comparable regardless of which calibration method was used, as long as internal standard correction was applied. The post-extraction fortified calibrant trueness for BG matched the collaborative study results with enhanced recoveries (129–163%); however, in the single-laboratory validation, very low recoveries were found for BG using solvent based calibrants (0–64%). From the results of both studies, and the procedure described by First Action **2012.25** (8), it is clear that acceptable method trueness for all analytes is achieved only when extracted matrix calibrants with internal standard correction are used for quantitative analysis.

Qualitative Results

Analyte identification was achieved by comparison of peak area ratios of the qualitative:quantification product ion transitions of test samples to the average value of the ratios obtained from extracted calibrant samples (0.25–5 µg/L). These results are summarized in Table 6. Acceptability criteria for both the EU (±20–50%, based on ratio found; 10) and the FDA (±10% absolute; 11) were applied to the data, and the results were compared. It is interesting to note that while there were individual cases where one or the other approach provided higher identification percentages, on the whole, the two approaches provided comparable results. Evaluation of retention times for identification revealed two laboratories that had some difficulty in meeting the stricter EU standard (±2.5%) on a total of six or 13 samples, respectively. All samples, however, met the FDA retention time standard (±5%).

In general, identification was successful for the overwhelming majority of samples. Blank samples did occasionally meet identification criteria, particularly for CV and LCV, as evidenced by the higher percentage of identifications listed for those blank samples (Table 6). Although these samples did meet the requirement of having signals greater than three times the instrument noise, the calculated concentrations for most blank samples was below 0.05 µg/kg. Thirteen of the 84 blank samples met identification criteria and had a calculated analyte concentration >0.05 µg/kg; calculated concentrations for those individual blank samples are reported in Table 6. Five of those blank samples have analytes with concentrations at or above the MDL for the particular analyte/matrix pair: 0.08 µg/kg LMG in salmon, 0.14 µg/kg CV in salmon, 0.30 µg/kg CV in shrimp, and 0.12 and 0.13 µg/kg LCV in catfish. None of the blank samples have analyte concentrations that exceed the CCα for the analyte/matrix pair. In general, concentrations of the identified analytes in the blank samples are well below the 1 µg/kg level of concern. True false-positive samples may be a result of instrument carryover or trace contamination with ink from commonly used laboratory marking pens. For best results, it is advisable to inject water samples between test samples to identify and minimize interference (8), and to avoid the use of laboratory marking pens when labeling samples.

Use for Screening

The authors of the first action method proposed that this method could be used as a screening method by estimating the concentration of residues in an unknown sample by comparison to a single point extracted matrix calibrant spiked at 0.5 µg/kg. In that analytical strategy, unknown samples that yielded corrected peak areas greater than those generated for the 0.5 matrix calibrant would require a secondary analysis with a full calibration curve (8). From the results of the 14 participating laboratories, peak area data (internal standard corrected) for each 0.5 µg/kg extracted matrix calibrant was tabulated and compared to the appropriate (matching analyte and matrix) corrected peak area for the 10 blinded unknown samples analyzed by each laboratory. The percentage of blinded samples that yielded peak areas greater than the peak area of the 0.5 µg/kg calibrant is summarized in Table 7. None of the negative control samples yielded peak areas greater than the 0.5 µg/kg calibrant, and all of the 1.75 µg/kg fortified samples had responses greater than the 0.5 µg/kg calibrant. For the 0.42 µg/kg fortified samples, 14% (58 of the 420 analyte measurements; five analytes × three matrixes × duplicate samples × 14 laboratories) yielded peak areas greater than the 0.5 µg/kg calibrant. Of these, 22 of the analyte measurements (5%) yielded peak areas >20% of the 0.5 µg/kg calibrant, corresponding to concentrations of 0.62 to 1.14 µg/kg based on the single calibrant estimation. Table 7 highlights the screen results/analyte; however, it should be noted that one sample often yielded incorrect screen results for more than one residue. For example, catfish at the 0.42 µg/kg level yielded several samples

with responses above the 0.5 µg/kg calibrant for three or four residues. At the 0.90 µg/kg concentration, eight analytes (2%) generated peak areas below the response for the 0.5 extracted matrix calibrant. Based on the single point calibrant, these eight would yield residue concentrations in the range of 0.39 to 0.48 µg/kg. Some incurred samples were screened below the 0.5 µg/kg extracted matrix calibrant, but most of these had mean residue concentrations below 0.4 µg/kg, making it reasonable that these samples would be screened below the 0.5 µg/kg matrix calibration level. One MG in shrimp "incurred" sample yielded an estimated concentration of 0.44 µg/kg, yet the MG incurred shrimp mean concentration was 0.71 µg/kg.

Recommendation

This method has provided clearly acceptable results at and below the level of interest (1 µg/kg) for the triphenylmethane dyes studied. The method accuracy was excellent with trueness generally ≥90%, precision generally ≤10% RSDr, and HorRat values <1. The Study Directors recommend this method for acceptance as Final Action status.

Acknowledgments

We thank John Phillips (U.S. Department of Agriculture, Agricultural Research Service, Wyndmoor, PA) for performing the statistical analysis for this study.

We also acknowledge the following individuals from the FDA for their technical assistance with the preparation of the study samples: Sherri Turnipseed and Joseph Storey (Animal Drugs Research Center, Office of Regulatory Affairs, Denver Laboratory, Denver, CO); Christine Casey, Edward Iannuzzi, Ryan Stadtmuller, Julie Lillehaugen, Andrea Heise, Zachary Miller, Ashleigh Gillaspie, and Mark Madson (Office of Regulatory Affairs, Denver Laboratory, Denver, CO); and Eric Evans and Scott Frobish (Center for Veterinary Medicine Aquaculture Program, Laurel, MD).

We thank Sherri Turnipseed and Steve Lehotay (USDA-ARS, Wyndmoor, PA) for assistance with design of the study protocol, Kai Klocke for assistance with programming and data transformation, and Tom Phillips (Maryland Department of Agriculture, Annapolis, MD) for additional statistical review.

Finally, we acknowledge the following collaborators for their participation in this study:

Mark Crosswhite and Walter Hammack, Florida Department of Agriculture and Consumer Services, Division of Food Safety, Chemical Residue Laboratory, Tallahassee, FL

Christopher K. Lam and Mary Cumming, Tennessee Department of Agriculture, Ivy Laboratory, Nashville, TN

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Alan R. Lightfield and Steven J. Lehotay, U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA

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AOAC AWARDS-**2016** METHOD OF THE YEAR (ERP Chair Nomination Form)

Submission Date	2016-04-29 12:41:24
Name	Joe Boison
Panel Name	Determination of 3 triphenylmethane dye residues and their metabolites in aquaculture products by LC-MS/MS
E-mail	joe.boison@inspection.gc.ca
Date	04-29-2016
AOAC Method Number	2012.25
Method Name	residues of three triphenylmethane dyes and theri metabolites in aquaculture products y LC-MS/MS
Rationale (based on selection criteria)	The Single Laboratory validated Method 2012.25 was recommended to First Action Official Method Status following the face-to-face ERP meeting held in Las Vegas September/October 2012. Following that the scope of the method was expanded from just fish muscle tissue to include shrimp, tilapia, catfish, and salmon without significant changes to the method procedure. The scope expanded method was subjected to a 14 laboratory collaborative study and the feedback from the participating laboratories were all highly positive with the added recommendation that the method will provide a needed gap in the residue monitoring control program for most countries for residues of these dyes which have been recommended by the Codex Alimentarius Commission not to be used in food animal production, especially aquaculture. The most significant asset of this study was that because the analysis and interpretation of the collaborative study data were so well handled by the authors, a consensus decision to recommend this First to Final Action was recommended by the Expert Review Panel through a teleconference. As chair of several ERPs in the past 4 years, I think this is the FIRST-to-FINAL Official Status method that has been so recommended following a teleconference call instead of a face-to-face. I strongly recommend that the role played by the ERP be recognized and the authors of the collaborative study together with the authors of the SLV be cited for this notable achievement.

MICROBIOLOGICAL METHODS

Evaluation of VIDAS® UP *Salmonella* (SPT) Assay for the Detection of *Salmonella* in a Variety of Foods and Environmental Samples: Collaborative Study

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The VIDAS® UP *Salmonella* (SPT) uses recombinant phage proteins to detect *Salmonella* species in human and animal food products and production environmental samples after 18–26 h of enrichment. The VIDAS SPT assay is performed with the automated VIDAS or mini-VIDAS instruments. The VIDAS SPT method was compared in a multilaboratory collaborative study to the U.S. Department of Agriculture/Food Safety and Inspection Service-*Microbiology Laboratory Guidebook* (USDA/FSIS-MLG) 4.05 (2011) *Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg and Catfish Products* reference method following the current AOAC guidelines. A total of 15 laboratories representing government, academia, and industry throughout the United States participated. One matrix, raw ground beef, was analyzed using two different test portion sizes, 25 and 375 g. Each test portion was artificially contaminated with *Salmonella* at three inoculation levels, an uninoculated control level (0 CFU/test portion), a low inoculum level (0.2–2 CFU/test portion), and a high inoculum level (2–5 CFU/test portion). In this study, 1656 unpaired replicate samples were analyzed. Of those unpaired replicates, 476 were presumptive positive by the VIDAS method, with 475 confirmed positive by the traditional confirmation procedures and 476 confirmed positive by an alternative confirmation procedure. There were 411 confirmed positive replicates by the USDA/FSIS-MLG reference method. Statistical analysis was conducted according to the probability of detection (POD). For the low-level 375 g test portions, the following dLPOD values, with 95% confidence intervals, were obtained: 0.01 (–0.12, +0.15) for samples confirmed following the traditional confirmation; 0.02 (–0.18, +0.2) for samples confirmed following traditional confirmation on IBISA and ASAP; and 0.03 (–0.18, +0.24) for samples confirmed following the alternative confirmation on IBISA and ASAP. For the low-level 25 g test portions, the following dLPOD values, with 95% confidence intervals, were obtained: 0.41, (0.32, +0.49) for samples confirmed following the traditional confirmation, the traditional confirmation on IBISA and ASAP, and the alternative confirmation on IBISA and ASAP. With 0.0 within the confidence intervals for the 375 g test portions, there was no statistically significant difference in the number of positive samples detected by the VIDAS SPT method and the USDA/FSIS-MLG method at the 0.05 level. For the 25 g test portions, a statistically significant difference was observed between the VIDAS SPT method and the reference method for the low inoculum level, where the VIDAS SPT method recovered a higher number of positive results than the reference method. It is recommended that the VIDAS SPT method with the optional ASAP and IBISA agar confirmation method be adopted for Official First Action status for the detection of *Salmonella* in a variety of foods and environmental samples.

Submitted for publication February 2013.
The recommendation was approved by the Methods Committee on Microbiology as First Action.
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Appendixes are available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI: 10.5740/jaoacint.CS2013_01

Salmonellosis, the foodborne illness caused by the bacterium *Salmonella,* has been linked to numerous foodborne outbreaks associated with a wide range of products, such as meat, poultry, eggs, dairy products, fresh produce, spices, sauces, peanut butter, and chocolate (1). Taking up to 5 days to confirm, the detection of *Salmonella* species can be time-consuming and expensive for food manufacturers. With more than 2500 different serovars, *Salmonella* are antigenically complex due to variations in their lipopolysaccharide and flagellar protein antigens (1). The VIDAS UP *Salmonella* (SPT) assay, an automated enzyme phage-ligand-based assay for the detection of *Salmonella* in food and environmental samples, uses recombinant phage proteins to detect both motile and

nonmotile *Salmonella.* The assay is performed in the automated VIDAS instrument.

The VIDAS SPT assay uses a primary enrichment (prewarmed to 42±1°C for 375 g samples), along with a proprietary supplement (SPT supplement). After 18–24 h of enrichment (22–26 h for 375 g samples), *Salmonella* detection is performed by the VIDAS SPT test. The new enrichment method eliminates the need for secondary enrichments [Tetrathionate Hanja (TTH), Rappaport-Vasilliadis (RV), and SX2 broths]. Negative results and presumptive positive results are available the day after enrichment.

Prior to the collaborative study, the VIDAS SPT method was validated according to AOAC guidelines for harmonized *Performance Tested Method*^{SM} (PTM) studies (2). The purpose of this study was to demonstrate that the VIDAS SPT method could detect *Salmonella* in a variety of foods and environmental surfaces as claimed by the manufacturer. For the VIDAS SPT PTM evaluation, 17 matrixes were tested using buffered peptone water (BPW) plus *Salmonella* supplement enrichment protocol: raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), and stainless steel, plastic, and ceramic environmental surfaces. In a matrix extension evaluation conducted in February 2012, three additional foods were evaluated using BPW plus *Salmonella* supplement enrichment protocol: raw ground turkey (375 g), almonds (375 g), and chicken carcass rinsates (30 mL). One matrix, raw ground beef (375 g), was evaluated using a different enrichment protocol, BPW plus vancomycin, to allow for a single enrichment when the VIDAS SPT and *E. coli* Phage Technology (bioMérieux) assays were used.

All other PTM parameters (inclusivity, exclusivity, ruggedness, stability, and lot-to-lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method was awarded PTM certification No. 071101 on July 15, 2011 with a matrix extension approval on March 23, 2012.

This collaborative study compared the VIDAS SPT method to the U.S. Department of Agriculture/Food Safety and Inspection Service-*Microbiology Laboratory Guidebook* (USDA/FSIS-MLG) 4.05 (2011) *Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg and Catfish Products* method (3) for raw ground beef at two test portion sizes, 25 and 375 g.

Collaborative Study

Study Design

For this collaborative study, one matrix, raw ground beef (80% lean), was analyzed using two different test portion sizes, 25 and 375 g. The raw ground beef was obtained from local retailers and screened for the absence of *Salmonella* by the USDA/FSIS-MLG reference method prior to analysis. The screening indicated an absence of indigenous *Salmonella*. For analysis of the 25 g test portions, the raw ground beef was artificially contaminated with *Salmonella* Enteritidis ATCC 13076 and with *Salmonella* Montevideo ATCC 8387 for the analysis of the 375 g test portions. There were two inoculation levels: a high inoculation level of approximately 2–5 CFU/test portion and a low inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions were also included for each matrix at 0 CFU/test portion.

Twelve replicate samples from each of the three inoculation levels of product were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by VIDAS SPT and the USDA/FSIS-MLG reference method due to different sample enrichments for each method. For both test portion sizes, collaborators were sent an additional 30 g test portion and instructed to conduct a total aerobic plate count on the day samples were received in order to determine the total aerobic microbial load in the matrix.

A detailed collaborative study packet outlining all necessary information related to the study, including media preparation, method-specific test portion preparation, and documentation of results, was sent to each collaborating laboratory before initiation of the study.

Preparation of Inocula and Test Portions

The *Salmonella* cultures used in this evaluation were propagated in 10 mL Brain Heart Infusion broth from a frozen stock culture held at –70°C at Q Laboratories, Inc. The broth was incubated for 18–24 h at 35±1°C. Appropriate dilutions were prepared based on previously established growth curves for both low and high inoculation levels, resulting in fractional positive outcomes for at least one level. For both test portion sizes, a bulk lot of the raw ground beef was inoculated with a liquid inoculum and mixed thoroughly by hand-kneading to ensure even distribution of microorganisms. The raw ground beef was inoculated on the day of shipment so that all test portions would have been held for 96 h by the day testing was initiated. For the analysis of the 25 g test portions, the bulk lot of test material was divided into 30 g portions for shipment to the collaborators. For the analysis of the 375 g test portions, 25 g of inoculated test product was mixed with 350 g of uninoculated test product for shipment to the collaborators for analysis by the VIDAS SPT method. Collaborators received 30 g portions for analysis by the USDA/FSIS-MLG method. To determine the level of *Salmonella* spp. in the raw ground beef, a five-tube MPN was conducted on the day of initiation of analysis. From both the high and low inoculated batches of raw ground beef, five 100 g test portions, five 25 g test portions, and five 10 g test portions were analyzed using a 1:10 dilution with BPW. The most probable number (MPN) and 95% confidence intervals were calculated from the high, medium, and low levels using the AOAC MPN Calculator (www.lcftld.com/customer/LCFMPNCalculator.exe; 4).

Confirmation of the samples was conducted according to the USDA/FSIS-MLG 4.05 reference method.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded, three-digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transport Association. Upon receipt, samples were held by the collaborating laboratory at refrigeration

Table 2013.01A. Summary of results for the detection of *Salmonella* spp. in raw ground beef (25 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/144	144/144	144/144	0/144	144/144	144/144	0/144	144/144	144/144
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	1.00 (+0.97, +1.00)	1.00 (+0.97, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_L[e]	0.00 (0.00 +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)
s_R[f]	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)
P-value	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
Candidate confirmed positive/total samples analyzed	0/144	143/144	144/144	0/144	143/144	144/144	0/144	143/144	144/144
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.99 (+0.96, +1.00)	1.00 (+0.97, +1.00)
s_r	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.08 (+0.07, +0.16)	0.00 (0.00, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)	0.00 (0.00, +0.16)	0.00 (0.00, +0.03)	0.00 (0.00, +0.16)
s_R	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)	0.00 (0.00, +0.22)	0.08 (+0.08, +0.10)	0.00 (0.00, +0.22)
P-value	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000	1.0000	0.4368	1.0000
Positive reference samples/total samples analyzed	0/144	84/144	138/144	0/144	84/144	138/144	0/144	84/144	138/144
Reference POD	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)	0.00 (0.00, +0.03)	0.58 (+0.50, +0.67)	0.96 (+0.91, +0.98)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.19 (+0.17, +0.22)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.06 (+0.02, +0.13)
s_R	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)	0.00 (0.00, +0.22)	0.50 (+0.45, +0.52)	0.20 (+0.18, +0.24)
P-value	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179	1.0000	0.6298	0.0179
dLPOD (candidate vs reference)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (0.01, +0.09)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)	0.00 (–0.03, +0.03)	0.41 (+0.32, +0.49)	0.04 (+0.01, +0.09)
dLPOD (candidate presumptive vs candidate confirmed)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)	0.00 (–0.03, +0.03)	0.01 (–0.02, +0.04)	0.00 (–0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

temperature (3–5°C) until the following Monday when analysis was initiated. All samples were packed with cold packs to target a temperature of <7°C during shipment. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled "temperature control." Participants were instructed to obtain the temperature of this portion upon receipt of the package, document results on the Sample Receipt Confirmation form provided, and fax to the Study Director.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method for each test portion size. For both test portion sizes, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls for each evaluation). For the analysis of the 25 g test portions by the VIDAS SPT method, a 25 g portion was enriched with 225 mL BPW and homogenized for 2 min. *Salmonella* supplement (1 mL) was added to the enrichment, and the test portions were incubated for 18–24 h at 42 ± 1°C. For the 375 g test potions analyzed by the VIDAS SPT method, a 375 g portion was enriched with 1125 mL prewarmed (42 ± 1°C) bioMérieux BPW and homogenized for 2 min. *Salmonella* supplement (5 mL) was added to the enrichment, and test portions were incubated for 22–26 h at 42 ± 1°C.

After enrichment, samples were assayed by the VIDAS SPT method and confirmed using procedures outlined in the standard reference method by transferring an aliquot of the primary enrichment to secondary selective enrichment broths, TTH and RV. After incubation of the secondary selective enrichments, samples were struck to the selective agars specified in the USDA/FSIS-MLG and to two proprietary chromogenic agars, ASAP and IBISA. Presumptive positive samples from each agar were confirmed following the biochemical and serological procedures outlined in the USDA/FSIS-MLG.

An alternative confirmation for all VIDAS SPT samples was conducted by directly streaking an aliquot from the primary enrichment of each test portion to ASAP and IBISA chromogenic agar. Presumptive positive samples from each agar were confirmed following biochemical and serological procedures outlined in the USDA/FSIS-MLG method.

Both test portion sizes analyzed by the VIDAS SPT methods were compared to samples (25 g) analyzed using the USDA/FSIS-MLG reference method in an unpaired study design. Test portions of 25 g were enriched in BPW, homogenized for 2 min, and incubated at 35 ± 2°C for 24 ± 2 h. Samples were transferred to selective secondary enrichments and streaked to agars specified in the USDA/FSIS-MLG method. All positive test portions were biochemically confirmed by the API 20E biochemical test, AOAC *Official Method* **978.24** or the VITEK GN identification test, AOAC *Official Method* **2011.17**. Serological testing was also performed.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method, VIDAS SPT results, and the results for both the traditional and alternative confirmation of the VIDAS SPT samples on the data sheets provided. The data sheets were submitted to the Study Director at the end of each week of testing for analysis. The results of each test portion for each sample were compiled by the Study Director, and the qualitative VIDAS SPT results were compared to the reference method for statistical analysis. Data for each test portion size were analyzed using the probability of detection (POD) statistical model (5). If the confidence interval of a dLPOD did not contain zero, that would indicate a statistically significant difference between the VIDAS SPT method and the USDA/FSIS-MLG reference method at the 5% probability level.

AOAC Official Method 2013.01
Salmonella in a Variety of Foods
VIDAS® UP *Salmonella* (SPT) Method
First Action 2013

[Applicable to detection of *Salmonella* in raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), raw ground turkey (375 g), almonds (375 g), chicken carcass rinsates (30 mL), and stainless steel, plastic, and ceramic environmental surfaces.]

See Tables **2013.01A** and **B** for a summary of results of the interlaboratory study. For detailed results of the interlaboratory study, *see* Tables A–F in Appendix 1 on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

A. Principle

The VIDAS SPT method is for use on the automated VIDAS instrument for the detection of *Salmonella* receptors using the enzyme-linked fluorescent assay. The solid-phase receptacle (SPR) serves as the solid phase, as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Salmonella* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. The instrument performs all the assay steps automatically. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Salmonella* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Salmonella* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methylumbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methylumbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS SPT assay kit from bioMérieux Inc., Hazelwood, MO.

(**a**) *VIDAS or miniVIDAS automated immunoassay system.*

(**b**) *SPT reagent strips.*—60 polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents in Table **2013.01**.

(**c**) *SPR.*—60 SPRs coated with proteins specific for *Salmonella* receptors.

(**d**) *Standard.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—One vial (6 mL). Contains purified and inactivated *Salmonella* receptors + preservative + protein stabilizer.

(**f**) *Negative control solution.*—One vial (6 mL). Contains Tris-buffered saline (150 mmol/L)–Tween pH 7.6 + preservative.

(**g**) *Master lot entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test.

(**h**) *Package insert*.

(**i**) *Disposable pipet to dispense appropriate volumes.*

(**j**) *VIDAS Heat and Go.*—Available from bioMérieux, Inc.

(**k**) *Water bath (95–100°C) or equivalent system.*

(**l**) *Stomacher®-type bag with filter.*

(**m**) *Stomacher.*—Stomacher Lab Blender 400, available from Seward Medical (London, UK); Smasher, bioMérieux, Inc., or equivalent.

(**n**) *BPW.*—Available from bioMérieux, Inc.

(**o**) *Salmonella supplement.*—Available from bioMérieux, Inc.

(**p**) *Incubators.*—Capable of maintaining 42±1°C and 35±1°C.

(**q**) *Diagnostic reagents.*—Necessary for culture confirmation of assays. *See* AOAC *Official Method* **967.27**.

(**r**) *IBISA chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**s**) *ASAP chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**t**) *Vancomycin.*—Available from bioMérieux, Inc.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS SPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Mix standard, controls, and heated test portions well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS SPT package insert (refer to the following sections in the package insert: Warnings and Precautions and Waste Disposal).

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion in BPW using filter Stomacher bags to initiate growth of *Salmonella.* For 25 g test portions, add 225 mL BPW to each test portion and homogenize thoroughly for 2 min. For 375 g test portions, pre-warm BPW to 42±1°C, add 1125 mL to each test portion, and homogenize thoroughly for 2 min.

(**b**) After homogenization add *Salmonella* supplement to each test portion. For 25 g test portions, add 1 mL of *Salmonella* supplement, mix samples manually, and incubate for 18–24 h at 42±1°C. For 375 g test portions, add 5 mL of *Salmonella* supplement, mix samples manually, and incubate for 22–26 h at 42±1°C.

(**c**) After incubation, homogenize samples manually. If a water bath is used, transfer 2–3 mL enrichment broth into a tube. Seal the tube. Heat for 5±1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS SPT reagent strip. If the VIDAS Heat and Go is used, transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS SPT reagent strip. Heat for 5±1 min (*see* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and allow them to come to room temperature.

(**c**) Use one VIDAS SPT reagent strip and one VIDAS SPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "SPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the SPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, test sample identification, date and time, lot number, and expiration date of the reagent kit being used, each sample's RFV**,** test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of

the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A negative result has a test value less than the threshold (0.25) and indicates that the sample does not contain *Salmonella* spp. or contains *Salmonella* spp. at a concentration below the detection limit. A positive result has a test value equal to or greater than the threshold (≥0.25) and indicates that the sample may be contaminated with *Salmonella* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2012.01D**).

G. Confirmation

All positive VIDAS SPT results must be culturally confirmed. Confirmation should be performed using the non-heated enrichment broth stored between 2 and 8°C, and should be initiated within 72 h after the end of incubation at 42 ± 1°C. Presumptive positive results may be confirmed by isolating on selective agar plates such as IBISA or ASAP, or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in AOAC *Official Method* **967.27**. As an alternative to the conventional tube system for *Salmonella*, any AOAC-approved commercial biochemical kits may be used for presumptive generic identification of foodborne *Salmonella* as described in AOAC *Official Methods* **978.24**, **989.12**, **991.13**, and **2011.17**.

Results of Collaborative Study

In this collaborative study, the VIDAS SPT method was compared to the to the USDA/FSIS-MLG reference method for one food product, raw ground beef, at two different test portion sizes, 25 and 375 g. A total of 15 laboratories throughout the United States participated in this study, with 14 submitting data for each matrix, as presented in Table 1. Each laboratory analyzed 36 test portions for each method: 12 inoculated with a high level of *Salmonella*, 12 inoculated with a low level, and 12 uninoculated controls. For each test portion size, the actual level of *Salmonella* was determined by MPN determination on the day of initiation of analysis. Individual laboratory and sample results are presented in Tables 2–5. Tables **2013.01A** and **B** summarize the interlaboratory results for all foods tested, including POD statistical analysis (6). Detailed results for each laboratory are presented in Tables A–F of the Appendix.

Raw Ground Beef (25 g Test Portions)

Raw ground beef test portions were inoculated at a low and high level, and analyzed (Tables 2 and 3) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. Fourteen laboratories participated in the analysis of this matrix, and the results of 12 were included in the statistical analysis. Laboratory 8 reported that it was unable to confirm samples via serological testing and indicated that it did not conduct the alternative confirmation of the VIDAS SPT samples. Therefore, its results were not included in statistical analysis. Laboratory 12 produced a low-level presumptive positive result for one of its uninoculated control test portions, which could not be confirmed positive by the traditional reference method. Therefore, its results were not included in the statistical analysis. The MPNs obtained for this matrix, with 95% confidence intervals, were 1.10 CFU/test portion (0.49, 2.46) for the low inoculum level and 4.38 CFU/test portion (1.71, 11.20) for the high inoculum level.

Traditional Confirmation with Xylose-Lysine-Tergitol 4 (XLT4) and Brilliant Green Sulfa (BGS)

For the high inoculum level, all of the 144 test portions were reported positive by the VIDAS SPT method, with all portions confirming positive. For the low inoculum level, all 144 test portions were also reported as positive by the VIDAS SPT method, with 143 confirming positive, indicating one false unconfirmed positive result (Laboratory 6). For the uninoculated controls, none of the 144 samples produced a presumptive positive result by the VIDAS SPT method, and all samples confirmed negative. For test portions analyzed by the USDA/FSIS-MLG method, 138 out of 144 high and 84 out of 144 low inoculum test portions confirmed positive. For the uninoculated controls, none of the 144 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.41 (+0.32, +0.49) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method. A $dLPOD_{CP}$ of 0.01 (–0.02, +0.04) was obtained between presumptive and confirmed VIDAS SPT results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.04 (+0.01, +0.09) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method. A $dLPOD_{CP}$ of 0.00 (–0.03, +0.03) was obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Results of the POD statistical analysis are presented in Table **2013.01A**, and in appended Table A and Figure 1A and B.

Traditional Confirmation with IBISA and ASAP

For the high inoculum level, all 144 test portions were reported as positive by the VIDAS SPT method, with all confirming positive. For the low inoculum level, all 144 test portions were also reported as positive by the VIDAS SPT method, with 143 confirming positive. For the uninoculated controls, none of the 144 samples produced a presumptive positive result by the VIDAS SPT method with all samples confirming negative. For test portions analyzed by the USDA/FSIS-MLG method, 138 of the 144 high inoculum test portions and 84 out of 144 low inoculum test portions confirmed positive. For the uninoculated controls, none of the 144 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.41 (+0.32,

Table 2013.01B. Summary of results for the detection of *Salmonella* spp. in raw ground beef (375 g)

Method[a]	VIDAS SPT with traditional confirmation on BGSA and XLT4			VIDAS SPT with traditional confirmation on IBISA and ASAP[b]			VIDAS SPT with alternative confirmation on IBISA and ASAP[c]		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total samples analyzed	0/132	58/131	130/132	0/132	58/131	130/132	0/132	57/131	130/132
Candidate presumptive POD (CP)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.33, +0.54)	0.98 (+0.965, +1.00)
s_r[d]	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44. +0.52)	0.12 (+0.11, +0.16)
s_L[e]	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.26)	0.00 (0.00, +0.05)
s_R[f]	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.44, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.1551	0.5190	1.0000	0.1906	0.5190
Candidate confirmed positive/total samples analyzed	0/132	58/131	130/132	0/132	59/131	130/132	0/132	58/131	130/132
Candidate confirmed POD (CC)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.45 (+0.35, +0.55)	0.98 (+0.95, +1.00)	0.00 (0.00, +0.03)	0.44 (+0.34, +0.55)	0.98 (+0.95, +1.00)
s_r	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.12 (+0.11, +0.16)	0.00 (0.00, +0.16)	0.49 (+0.43, +0.52)	0.12 (+0.11, +0.16)
s_L	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.09 (0.00, +0.25)	0.00 (0.00, +0.05)	0.00 (0.00, +0.16)	0.10 (0.00, +0.27)	0.00 (0.00, +0.05)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.12 (+0.11, +0.14)
P-value	1.0000	0.1551	0.5190	1.0000	0.2060	0.5190	1.0000	0.1551	0.5190
Positive reference samples/total samples analyzed	0/132	57/132	132/132	0/132	57/132	132/132	0/132	54/132	131/132
Reference POD	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.43 (+0.35, +0.52)	1.00 (+0.97, +1.00)	0.00 (0.00, +0.03)	0.41 (+0.32, +0.50)	0.99 (+0.96, +1.00)
s_r	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.16)
s_L	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.00 (0.00, +0.18)	0.00 (0.00, +0.17)	0.00 (0.00, +0.16)	0.05 (0.00, +0.22)	0.00 (0.00, +0.04)
s_R	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.23)	0.00 (0.00, +0.23)	0.49 (+0.44, +0.52)	0.09 (+0.08, +0.10)
P-value	1.0000	0.6261	1.0000	1.0000	0.6261	1.0000	1.0000	0.3313	0.4338
dLPOD (C vs R)	0.00 (–0.03, +0.03)	0.01 (–0.12, +0.15)	–0.02 (–0.05, +0.02)	0.00 (–0.03, +0.03)	0.02 (–0.18, +0.22)	–0.02 (–0.05, +0.02)	0.00 (–0.03, +0.03)	0.03 (–0.18, +0.24)	–0.01 (–0.05, +0.03)
dLPOD (CP vs CC)	0.00 (–0.03, +0.03)	0.00 (–0.15, +0.15)	0.00 (–0.04, +0.04)	0.00 (–0.03, +0.03)	–0.01 (–0.15, +0.14)	0.00 (–0.04, +0.04)	0.00 (–0.03, +0.03)	–0.01 (–0.21, +0.23)	0.00 (–0.04, +0.04)

[a] Results include 95% confidence intervals.

[b] Traditional confirmation on ASAP/IBISA = secondary enrichments streaked onto IBISA and ASAP.

[c] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[d] Repeatability standard deviation.

[e] Among-laboratory standard deviation.

[f] Reproducibility standard deviation.

Table 2013.01C. Reagents included in 10-well reagent strip

Wells	Reagents (SPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): Buffer pH 7.8 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-buffered saline (150 mmol/L) – Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Salmonella* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA; 0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent; *see* VIDAS SPT package insert for more information.

+0.49) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method. A $dLPOD_{CP}$ of 0.01 (–0.02, +0.04) was obtained between presumptive and confirmed VIDAS SPT results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.04 (+0.01, +0.09) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method. A $dLPOD_{CP}$ of 0.00 (–0.03, +0.03) was obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.01A** and in appended Table B and Figure 1C and D.

Alternative Confirmation with IBISA and ASAP

For the high inoculum level, all 144 test portions were reported as positive by the VIDAS SPT method, with all test portions confirming positive. For the low inoculum level, all 144 test portions were also reported as positive by the VIDAS SPT method, with 143 confirming positive. For the uninoculated controls, none of the 144 samples produced a presumptive positive result by the VIDAS SPT method, and all samples confirming negative. For test portions analyzed by the USDA/FSIS-MLG method, 138 of the 144 high inoculum test portions and 84 of the 144 low inoculum test portions confirmed

Table 2013.01D. Interpretation of test

Test value threshold	Interpretation
<0.25	Negative
≥0.25	Positive

Table 1. Participation of each collaborating laboratory[a]

Lab	Raw ground beef (25 g test portions)	Raw ground beef (25 g test portions)[b]	Raw ground beef (375 g test portions)	Raw ground beef (375 g test portions)[b]
1	Y	Y	Y	Y
2	Y	Y	Y[c]	Y
3	Y	Y	Y	Y
4	Y	Y	Y	Y
5	Y	Y	Y[c]	Y[c]
6	Y	Y	Y	Y
7	Y	Y	Y	Y
8	Y[c]	Y[c]	Y[c]	Y[c]
9	Y	Y	Y	Y
10	Y	Y	Y	Y
11	Y	Y	Y	Y
12	Y[c]	Y[c]	Y	Y[c]
13	Y	Y	Y	Y
14	Y	Y	N	N
15	N	N	Y	Y

[a] Y = Collaborator analyzed the food type; N = collaborator did not analyze the food type.

[b] Results were confirmed following the alternative confirmation procedure.

[c] Results were not used in statistical analysis due to laboratory error, or uninoculated control test portions were confirmed as *Salmonella*.

positive. For the uninoculated controls, none of the 144 test portions confirmed positive.

For the low level inoculum, a $dLPOD_C$ value of 0.41 (+0.32, +0.49) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method A $dLPOD_{CP}$ of 0.01 (–0.02, +0.04) was obtained between presumptive and confirmed VIDAS SPT results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.04 (+0.01, +0.09) was obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated a statistically significant difference between the two methods. However, the VIDAS SPT method detected more positive samples than the USDA/FSIS-MLG reference method, indicating a higher level of sensitivity than the reference method A $dLPOD_{CP}$ of 0.00 (–0.03, +0.03) was obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2012.01A** and in appended Table C and Figure 1E and F.

Table 2. Individual collaborator results for raw ground beef (25 g test portions) using traditional confirmation[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
VIDAS SPT (traditional confirmation)																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–[b]	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–[c]	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
USDA/FSIS-MLG																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	–	+	–	–	–	–[d]	–	–	–	–	–	–	–	–
10	–	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12[c]	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a] + = Salmonella spp. were detected in samples; – = Salmonella spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Sample was presumptive positive on VIDAS SPT, but confirmed negative indicating a false-positive result.

[c] Results were not used in statistical analysis due to laboratory error.

[d] Sample was confirmed negative on XLT4 and BGS, but confirmed positive on ASAP agar.

Raw Ground Beef (375 g Test Portions)

Raw ground beef test portions were inoculated at a low and a high level, and analyzed (Tables 4 and 5) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. Fourteen laboratories participated in the analysis of this matrix; the results of 11 laboratories were included in the statistical analysis. Laboratory 8 reported that it was unable to confirm samples via serological testing and indicated that it did not conduct the alternative confirmation of the VIDAS SPT samples. Therefore, its results were not included in statistical analysis. Laboratory 5 detected the presence of *Salmonella* spp. in their reference method uninoculated control replicates; therefore, its results were not included for statistical analysis. Laboratory 2 detected the presence of *Salmonella* spp. in their VIDAS SPT replicates following the traditional confirmation procedure, and Laboratory 12 detected the presence of *Salmonella* spp. in the confirmation of VIDAS SPT replicates following the alternative confirmation procedure. Therefore, their results were not included in the statistical analysis for those categories. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.72 CFU/test portion

Table 3. Individual collaborator results for raw ground beef (25 g test portions) using alternative confirmation[a]

	High-level test portions												High-level test portions												High-level test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
VIDAS SPT (alternative confirmation)[b]																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–[c]	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–[c]	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
USDA/FSIS-MLG																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	–	+	–	–	–	–	–[b]	–	–	–	–	–	–	–
10	–	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
15	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a] += Salmonella spp. were detected in samples; – = Salmonella spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[c] Sample was presumptive positive on VIDAS SPT but confirmed negative indicating a false-positive result.

[d] Results were not used in statistical analysis due to laboratory error.

[e] Sample was confirmed negative on XLT4 and BGS but confirmed positive on ASAP agar.

(0.31, 1.67) for the low level and 2.19 CFU/test portion (0.94, 5.12) for the high level.

Traditional Confirmation with XLT4 and BGS

For the high level, 130 of 132 test portions were reported as positive by the VIDAS SPT method, with all portions confirming positive. For the low level, 58 of 131 test portions were reported as positive by the VIDAS SPT method with 58 test portions confirming positive. For the uninoculated controls, none of the 132 samples produced a presumptive positive result by the VIDAS SPT method, and all confirmed negative. For test portions analyzed by the USDA/FSIS-MLG method, all of the 132 high and 57 of 132 low inoculum test portions confirmed positive. For the uninoculated controls, no test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.01 (–0.12, +0.15) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.15, +0.15) were obtained

Table 4. Individual collaborator results for raw ground beef (375 g test portions) using traditional confirmation[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
VIDAS SPT (traditional confirmation)																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2[b]	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	+	–	–	–
3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–[c]	–	–	–	–	–	–	NA	–	–	–	–	–	–	–	–	–	–	–	–
5[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	+	–	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
USDA/FSIS-MLG																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2[b]	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5[b]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = Salmonella spp. were detected in samples; – =Salmonella spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Results were not used in statistical analysis due to laboratory error.

[c] Sample was confirmed negative using the reference method agars XLT4 and BGS, but confirmed positive on ASAP agar.

between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of –0.02 (–0.05, +0.02) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.04, +0.04) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.01B** and in appended Table D and Figure 2A and B.

Traditional Confirmation with IBISA and ASAP

For the high level, 130 of the 132 test portions were reported as positive by the VIDAS SPT method, with all portions confirming positive. For the low level, 58 of 131 test portions were reported as positive by the VIDAS SPT method, with 59 confirming positive. For the uninoculated controls, none of the 132 samples produced a presumptive positive result by the VIDAS SPT method and all

Table 5. Individual collaborator results for raw ground beef (375 g test portions) using alternative confirmation[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
VIDAS SPT (alternate confirmation)[b]																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–[c]	–	–	–	–	–	–	NA	–	–	–	–	–	–	–	–	–	–	–	–
5[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	+	–	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12[d]	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–[c]	–[c]	–[c]
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
USDA/FSIS-MLG																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
5[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Salmonella* spp. were detected in samples; – = *Salmonella* spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Alternative confirmation = direct streak of the primary enrichment onto IBISA and ASAP.

[c] Sample was confirmed negative using the reference method agars XLT4 and BGS, but confirmed positive on ASAP and IBISA agar.

[d] Results were not used in statistical analysis due to laboratory error.

samples confirmed negative. For test portions analyzed by the USDA/FSIS-MLG method, all of the 132 high and 57 of 132 low inoculum test portions confirmed positive. For the uninoculated controls, none of the 132 test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.02 (–0.18, +0.22) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of –0.01 (–0.15, +0.14) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of –0.02 (–0.05, +0.02) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.04, +0.04) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are

presented in Table **2013.01B** and in appended Table E and Figure 2C and D.

Alternative Confirmation with IBISA and ASAP

For the high level, 130 of 132 test portions were reported as positive by the VIDAS SPT method, with all portions confirming positive. For the low level, 57 of 131 test portions were reported as positive by the VIDAS SPT method, with 58 confirming positive. For the uninoculated controls, none of the 132 samples produced a presumptive positive result by the VIDAS SPT method, and all samples confirmed negative. For test portions analyzed by the USDA/FSIS-MLG method, 131 of 132 high and 54 of 132 low inoculum test portions confirmed positive. For the uninoculated controls, none of the 132 test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.03 (–0.18, +0.24) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of –0.01 (–0.21, +0.23) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of –0.01 (–0.05, +0.03) were obtained between the USDA/FSIS-MLG method and the VIDAS SPT method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.04, +0.04) were obtained between presumptive and confirmed VIDAS SPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.01B** and in appended Table F and Figure 2E and F.

IBISA and ASAP Chromogenic Agar

Results obtained from the IBISA and ASAP chromogenic agars were comparable to the results obtained from the XLT4 and BGS agars specified by the USDA/FSIS-MLG method. For the samples analyzed by the reference method, there were 412 positive results obtained from ASAP agar plates, compared to 411 positive results obtained from XLT4 and BGS agar plates. For samples analyzed by the VIDAS SPT method and confirmed following traditional procedures using IBISA and ASAP there were 476 positive results obtained from ASAP agar plates, compared to 475 positive results obtained from IBISA, XLT4 and BGS agar plates. For samples analyzed by the VIDAS SPT method and confirmed following the alternative procedure using IBISA and ASAP, there were 479 positive results obtained from IBISA and ASAP agar plates, compared to 475 positive results obtained from XLT4 and BGS agar plates.

Four uninoculated control samples produced positive results on the IBISA and ASAP chromogenic agar that were not detected on either the XLT4 or BGSA reference agars or during analysis with the VIDAS SPT assay. Because the *Salmonella* species was not detected on the two reference agar plates, the positive results produced by the chromogenic agar plates may be an artifact of cross-contamination or laboratory error.

Discussion

For this collaborative study, samples were analyzed at both 375 and 25 g test portions as required by the current AOAC guidelines, which require methods with more than one sample preparation or enrichment scheme to analyze one matrix per procedure.

For the analysis of 375 g test portions, no significant difference was observed using the POD statistical model in the number of positive results obtained between the two methods being compared using both the traditional and alternative confirmation procedures for the VIDAS SPT method. For the analysis of 25 g test portions, a significant difference was observed using the POD statistical model between the two methods for both the low and high levels of inoculation using both the traditional and alternative confirmation procedures, with more positive results obtained using the VIDAS SPT method, indicating a high level of sensitivity in the detection of the target analyte by the candidate method.

The results of the POD statistical analysis may indicate the high sensitivity of the VIDAS SPT assay. The VIDAS SPT showed a higher sensitivity than the reference method when test portions of the same size (25 g) were analyzed, and similar sensitivity to the reference method for test portions that were 15x larger (375 g VIDAS SPT test portions, compared to 25 g USDA/FSIS-MLG test portions).

No negative feedback was reported to the Study Directors from the collaborating laboratories with regard to the performance of the VIDAS SPT assay or the IBISA and ASAP chromogenic agar. Overall, the VIDAS SPT method recovered *Salmonella* in 475 test samples out of 826 samples analyzed, compared to 411 positive results out of 826 samples for the USDA/FSIS-MLG method. Only one unconfirmed positive result and no false-negative results were obtained using the VIDAS SPT method.

Recommendations

It is recommended that the VIDAS SPT method, with the optional ASAP and IBISA agar confirmation method, be adopted as Official First Action status for the detection of *Salmonella* in a variety of foods, including raw ground beef (25 and 375 g), processed American cheese (25 g), deli roast beef (25 g), liquid egg (25 g), peanut butter (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), raw cod (25 g), bagged lettuce (25 and 375 g), dark chocolate (375 g), powdered eggs (25 g), instant nonfat dry milk (25 and 375 g), ground black pepper (25 g), dry dog food (375 g), raw ground turkey (375 g), almonds (375 g), chicken carcass rinsates, and stainless steel, plastic, and ceramic environmental surfaces.

Acknowledgments

We extend our sincere thanks to the following collaborators for their dedicated participation in this study:

John Mills, bioMérieux Industry, Hazelwood, MO

Judy Nogle, U.S. Food and Drug Administration, San Francisco District Office, Alameda, CA

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Tom Lonczynski and Laura Drasher, HACCP, Mountain Top, PA
Jerry Lynn Pickett, Jacquelyn Adams, Samantha Liles, and John Clayborn, Tyson WBA Analytical, Springdale, AR
Sergio Montez, Food Safety Net Services, San Antonio, TX
Mary Bandu and Matt Oltman, Chestnut Laboratory, Springfield, MO
Leslie Thompson and Jodene Jurgens, Aegis, North Sioux City, SD
Hesham Elgaali, Indiana State Department of Health, Food and Dairy Microbiology Division, Indianapolis, IN
Kristi McCallum and Dominika Kondratko, Colorado Department of Agriculture, Inspection and Consumer Services, Biochemistry Laboratory, Denver, CO
Yanyan Huang, ConAgra Foods, Microbiology and Microphysical Laboratory, Omaha, NE
Katie Goetz, Q Laboratories Inc., Cincinnati, OH

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FOOD BIOLOGICAL CONTAMINANTS

Detection of *Salmonella* species in a Variety of Foods by the DuPont™ BAX® System Real-Time PCR Assay for *Salmonella*: First Action 2013.02

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A multilaboratory study was conducted to evaluate the ability of the DuPont™ BAX® System Real-Time PCR Assay for *Salmonella* to detect the target species in a variety of foods and environmental surfaces. Internal validation studies were performed by DuPont Nutrition & Health on 24 different sample types to demonstrate the reliability of the test method among a wide variety of sample types. Two of these matrixes—pork and turkey frankfurters and pasteurized, not-from-concentrate orange juice without pulp—were each evaluated in 14 independent laboratories as part of the collaborative study to demonstrate repeatability and reproducibility of the internal laboratory results independent of the end user. Frankfurter samples were evaluated against the U.S. Department of Agriculture, Food Safety and Inspection Service reference method as a paired study, while orange juice samples were evaluated against the U.S. Food and Drug Administration reference method as an unpaired study, using a proprietary media for the test method. Samples tested in this study were artificially inoculated with a *Salmonella* strain at levels expected to produce low (0.2–2.0 CFU/test portion) or high (5 CFU/test portion) spike levels on the day of analysis. For each matrix, the collaborative study failed to show a statistically significant difference between the candidate method and the reference method using the probability of detection statistical model.

Received December 13, 2013.
The method was approved by the Expert Review Panel for Food Biological Contaminants as First Action.
The Expert Review Panel for Food Biological Contaminants invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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Appendices are available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI: 10.5740/jaoacint.13-407

Salmonella is a leading cause of foodborne illness. The low infectious dose of the bacterium makes it critical to detect even low concentrations of the *Salmonella* in foods. Additionally, the presence of high concentrations of closely related nonpathogenic bacteria create the need for highly accurate methodologies. Traditionally, laboratories concerned with detection of *Salmonella* screened food samples with culture methods, such as those provided by the U.S. Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) and the U.S. Food and Drug Administration (FDA), which require several days to detect and confirm *Salmonella*. Rapid methods of screening for *Salmonella* have been developed, but these generally require 2 days of enrichment. By contrast, the DuPont™ BAX® System detects the pathogen less than 90 min after enrichment, and the DNA-based results are both reliable and reproducible, leading to quicker release of cleared product.

The BAX System Real-Time PCR Assay for *Salmonella* was certified by the AOAC Research Institute in August 2012 and designated *Performance Tested Method*℠ (PTM) No. 081201. No significant differences were reported for detection of *Salmonella* in the matrixes tested when comparing the BAX System method results to the standard reference culture procedures described in the USDA-FSIS *Microbiology Laboratory Guidebook* (MLG; 1), FDA *Bacteriological Analytical Manual* (BAM; 2), and Health Canada *Compendium of Analytical Methods* (HC CAM; 3). The matrixes validated in the PTM study included raw ground beef (85% lean, 25 and 375 g), chicken carcass rinse, cream cheese (34% fat), fresh bagged lettuce, dry pet food, and stainless steel. Inclusivity testing demonstrated that the BAX System method was reactive with 317 *Salmonella* isolates, representing over 100 different serotypes. The test method did not detect 37 different non-*Salmonella* strains tested (Appendix 1; *see* appendixes on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac). After the PTM approval was achieved, a procedure change was applied to this validation to incorporate an eight-cycle increase in processing time in the BAX System Q7 instrument (Appendix 2).

Following the completion of the PTM study, a precollaborative study was conducted on an additional 18 matrixes, including

ground beef with soy (85% lean), beef trim, frankfurters (beef), shrimp, ground turkey, chicken wings, dried eggs (whole, powdered), shell eggs, frozen peas, orange juice, instant nonfat dry milk, ice cream (12% fat), peanut butter (52% fat), cocoa (unsweetened), white pepper, milk-based infant formula, ceramic tile, and plastic surfaces. The results obtained using the test method indicate no statistical difference with the reference method when compared to the corresponding reference method results (Appendix 3).

In addition, two of the precollaborative study matrixes—frankfurters (pork plus turkey) and orange juice (pasteurized not-from-concentrate)—were evaluated in a total of 15 independent laboratories as part of the collaborative study to demonstrate repeatability and reproducibility of the internal laboratory results independent of the end user. The results obtained using the BAX System method indicate no statistical difference when compared to the corresponding reference method results.

Collaborative Study

Study Design

Collaborators analyzed two representative matrixes (pork and turkey frankfurters and pasteurized, not-from-concentrate orange juice without pulp), 12 replicate test portions from each of three contamination levels (low, high, and uninoculated), comparing the performance of the BAX System Real-Time PCR Assay for *Salmonella* to appropriate reference culture methods. A total of 15 laboratories participated in the study, with 14 laboratories reporting data for each matrix. Each collaborator received instructions for performing the study and required materials prior to the start of the study. If necessary, training on the BAX System was provided to laboratory personnel by a DuPont representative.

The collaborative study was conducted in accordance with the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, Appendix J (4). Frankfurter samples were evaluated against the USDA-FSIS MLG reference method as a paired study, as the test and reference method enrichment protocols are identical. Orange juice samples were evaluated against the FDA-BAM reference method as an unpaired study, as the BAX System method uses enrichment in proprietary media. Estimates of repeatability, reproducibility, and probability of detection (POD) were evaluated.

Preparation of Inocula and Test Portions

Sample product was obtained from a local retail outlet and screened by the organizing laboratory to identify any naturally contaminating *Salmonella* and determine a total aerobic plate count. For each sample type, five analytical size portions (25 g for orange juice and 325 g for frankfurters) were screened for *Salmonella* using the appropriate reference method. Although naturally contaminated samples would have been preferred, all samples tested returned negative results for *Salmonella*. Therefore, each sample matrix was artificially inoculated with a different serovar of *Salmonella* for use in this study.

Portions of each sample type were inoculated at levels that on the day of initiation of analysis produced a high spike level (POD approximately 1.0 or approximately 5 CFU/test portion) and a low spike level (POD 0.25–0.75 or 0.2–2.0 CFU/test portion). Additional matrix was left uninoculated to serve as negative controls.

To inoculate frankfurter samples, a pure colony of *Salmonella* Typhimurium was transferred from Trypticase Soy agar with 5% sheep's blood (SBA) into Brain Heart Infusion (BHI) broth and incubated at 35°C for 18–24 h. The inoculum was heat stressed in a 55°C water bath for 10 min to obtain a percent injury of approximately 70% as determined by plating onto selective Xylose Lysine Desoxycholate (XLD) agar and nonselective TSA. Four portions of equal size were inoculated drop-wise with an 18–22 h culture of the target organism, and then homogenized by hand. All four portions were combined one at a time into a single container, homogenizing the bulk material after each portion was added. The bulk lot was separated into two sampling containers and 40 samples (20 for each method) weighing 25 g each were removed from each container. Each 25 g sample was combined with 300 g uncontaminated matrix to create 325 g test portions. The remaining spiked matrix was rehomogenized by combining the material from both containers into one and mixing thoroughly for the purposes of maintaining an even distribution of the organism.

To inoculate orange juice, a pure colony of *Salmonella* Hadar was transferred from SBA into BHI broth containing 1% glucose and incubated at 35°C for 18–24 h. This stress protocol resulted in a percent injury of approximately 60% (as determined by plating onto selective XLD agar and nonselective TSA). The inoculum was added drop-wise to a bulk quantity of orange juice to reach the desired contamination level, and then mixed to achieve equal distribution of the inoculum throughout. This spiked bulk quantity was divided into 25 mL test portions for analysis.

Test Portion Distribution

All test portions were randomized and blind-coded by the organizing laboratory, then shipped overnight to each collaborating laboratory and maintained at 2–8°C until they were analyzed. The total hold time of samples was 48 h for frankfurters and 96 h for orange juice, including shipment time to each participating laboratory. On the first day of test sample analysis, a 5-tube, 3-level most probable number (MPN) estimation of contamination levels was conducted by the organizing laboratory using the appropriate reference method. The Least Cost Formulations, Ltd (Norfolk, VA) MPN Calculator-Version 1.6 (5) was used to determine the MPN values and 95% confidence intervals. The MPN is reported for each level of each matrix in Appendix 4, Tables 1–6 as MPN/test portion with 95% confidence intervals.

Test Portion Analysis

For testing frankfurters, each collaborator received 12 low-spike, 12 high-spike, and 12 uncontaminated 325 g test portions, blind-coded so that the contamination level was unknown to the collaborator. Approximately one-third to one-half of 2925 ± 58.5 mL of sterile buffered peptone water (BPW) was added to each portion, and each portion was homogenized approximately 2 min. The remainder of the

2925 mL BPW was added, and samples were incubated at 35°C for 18–24 h. For the test method, samples were tested directly from the BPW enrichment using the BAX System method. For the USDA-FSIS MLG reference method, 0.5 mL aliquots of each portion were transferred to 10 mL tetrathionate (TT) Hajna broth, and 0.1 mL sample was added to 10 mL modified Rappaport-Vassiliadis (mRV) broth. All secondary enrichments were incubated at 42 ± 0.5°C for 22–24 h (or in a water bath for 18–24 h). Secondary enrichments were streaked to brilliant green sulfa and either double modified lysine iron agar (LIA) or xylose lysine Tergitol™ 4 agar plates and incubated 35 ± 2°C for 18–24 h. Isolated colonies were transferred to triple sugar iron (TSI) agar and LIA slants and incubated 35 ± 2°C for 22–26 h. *Salmonella* colonies were confirmed using serological (Somatic O and poly H agglutination) and biochemical procedures according to USDA-FSIS MLG.

For testing orange juice, each collaborator received 12 low-spike, 12 high-spike, and 12 uncontaminated 25 mL test portions blind-coded so that the contamination level was unknown to the collaborator. For the test method, samples were swirled with 225 mL BAX System MP media and incubated at 39–42°C for 22–26 h, then secondary enrichment was performed by transferring 10 µL primary enrichment to 500 µL prewarmed (37°C) BHI broth. Secondary enrichments were incubated at 37°C for 3 h, then tested with the BAX System method. For the FDA-BAM reference method, portions were swirled with 225 mL Universal Preenrichment Broth (UPB) and incubated at 35°C for 22–26 h. After primary enrichment, 1 mL of each enriched portion was transferred to 10 mL TT broth and 0.1 mL was transferred to 10 mL RV broth. RV tubes were incubated at 42 ± 0.2°C for 22–26 h using a circulating, thermostatically controlled water bath. TT tubes were incubated at 35 ± 2°C for 22–26 h. Secondary enrichments were streaked to bismuth sulfite, XLD, and Hektoen enteric agar plates and incubated at 35°C for 22–26 h. Isolated colonies were transferred to TSI and LIA slants and incubated 35 ± 2°C for 22–26 h. *Salmonella* colonies were confirmed using serological and biochemical procedures according to FDA-BAM.

Statistical Analysis

Data analysis was performed using each of the metrics below according to the format described in the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* and using the Least Cost Formulations, Ltd, AOAC Binary Data Interlaboratory Study Workbook (6).

For the purposes of this evaluation, POD was defined as the number of positive outcomes divided by the total number of trials. POD was estimated with a 95% confidence interval for each of the following levels: candidate presumptive results (POD_{CP}); candidate confirmatory results (POD_{CC}); candidate method results based on the presumptive and confirmatory results (POD_C); and reference method results (POD_R).

Candidate presumptive and confirmatory results were compared by determining the difference between the POD (dLOPD) values for each matrix and concentration ($dLPOD_{CP} = POD_{CP} - POD_{CC}$). If the confidence interval of a dLPOD does not contain zero, then the difference is statistically significant at the 5% level.

Candidate and reference method results were compared by determining the difference in POD values between the candidate and reference methods for each matrix and concentration ($dLPOD_C = POD_C - POD_R$). If the confidence interval of a dLPOD does not contain zero, then the difference is statistically significant at the 5% level.

AOAC Official Method 2013.02
Salmonella species in a Variety of Foods and Environmental Surfaces
BAX® System Real-Time PCR Assay for *Salmonella*
First Action 2013

[Applicable to the detection of *Salmonella* in a variety of foods, including raw ground beef (25 and 375 g), ground beef with soy (25 and 325 g), beef trim (25 and 325 g), frankfurters (325 g), shrimp (25 g), ground turkey (25 g), chicken wings (25 g), poultry rinse (30 mL), whole powdered (dried) eggs (25 g), shell eggs (1000 mL), fresh bagged lettuce (25 g), frozen peas (25 g), orange juice (pasteurized; 25 mL), cream cheese (25 g), nonfat dry milk (25 g), ice cream (25 g), peanut butter (25 g), cocoa (25 g), white pepper (25 g), milk-based infant formula (25 mL), and dry pet food (375 g), and on stainless steel, ceramic tile, and plastic surfaces.]

See Table **2013.02** for a summary of results of the collaborative study. *See* Appendix 4, Tables 1–6 for detailed results of the collaborative study.

Caution: Kits.—The reagents used in the BAX System should pose no hazards when used as directed. Dispose of lysate, PCR mixture, and other waste according to your site practices.

Cycler/detector.—Only qualified laboratory personnel should operate the cycler/detector. Do not attempt to repair the instrument. Live power may still be available inside the unit even when a fuse has blown or been removed. Refer to the BAX System User Guide for maintenance procedures when cleaning the unit or changing a fuse. The heating block can become hot enough during normal operation to cause burns or cause liquids to boil. Wear safety glasses or other eye protection at all times during operation.

Enrichment broths.—All enrichment broths may contain varying pathogens whether they contain *Salmonella* or not and thus should be sterilized and disposed of using proper procedures following any culture-based confirmatory steps.

Reference cultures.—When handling reference *Salmonella* cultures, always follow appropriate biosafety containment procedures as provided by your standard laboratory site practices, Centers for Disease Control and Prevention (CDC), or Canadian Pathogen Safety Data Sheets and Risk Assessment.

A. Principle

The DuPont™ BAX System uses the polymerase chain reaction (PCR) to amplify a specific fragment of bacterial

Table 2013.02. POD summary of results for the BAX System Real-Time PCR Assay for *Salmonella*

Method[a]	Orange juice (25 g)			Frankfurters (325 g)		
	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	0/168	94/168	168/168	0/168	87/168	168/168
Candidate presumptive POD (CP)	0.00 (0.00, +0.02)	0.56 (+0.48, +0.64)	1.00 (+0.98, +1.00)	0.00 (0.00, +0.02)	0.52 (+0.44, +0.60)	1.00 (+0.98, +1.00)
s_r[b]	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.51 (+0.46, +0.52)	0.00 (0.00, +0.15)
s_L[c]	0.00 (0.00, +0.15)	0.00 (0.00, +0.18)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.00 (0.00, +0.16)	0.00 (0.00, +0.15)
s_R[d]	0.00 (0.00, +0.21)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.21)	0.00 (0.00, +0.21)	0.51 (+0.46, +0.52)	0.00 (0.00, +0.21)
P-value	1.0000	0.4901	1.0000	1.0000	0.6831	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/168	92/168	168/168	0/168	85/168	168/168
Candidate confirmed POD (CC)	0.00 (0.00, +0.02)	0.55 (+0.47, +0.62)	1.00 (+0.98, +1.00)	0.00 (0.00, +0.02)	0.51 (+0.43, +0.58)	1.00 (+0.98, +1.00)
s_r	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)
s_L	0.00 (0.00, +0.15)	0.00 (0.00, +0.17)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.00 (0.00, +0.17)	0.00 (0.00, +0.15)
s_R	0.00 (0.00, +0.21)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.21)	0.00 (0.00, +0.21)	0.50 (+0.46, +0.52)	0.00 (0.00, +0.21)
P-value	1.0000	0.5903	1.0000	1.0000	0.5570	1.0000
Positive reference samples/total No. of samples analyzed	0/168	88/168	168/168	0/168	85/168	168/168
Reference POD	0.00 (0.00, +0.02)	0.52 (+0.45, +0.60)	1.00 (+0.98, +1.00)	0.00 (0.00, +0.02)	0.51 (+0.43, +0.58)	1.00 (+0.98, +1.00)
s_r	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.15)
s_L	0.00 (0.00, +0.15)	0.00 (0.00, +0.18)	0.00 (0.00, +0.15)	0.00 (0.00, +0.15)	0.00 (0.00, +0.17)	0.00 (0.00, +0.15)
s_R	0.00 (0.00, +0.21)	0.50 (+0.45, +0.52)	0.00 (0.00, +0.21)	0.00 (0.00, +0.21)	0.50 (+0.46, +0.52)	0.00 (0.00, +0.21)
P-value	1.0000	0.5021	1.0000	1.0000	0.5570	1.0000
dLPOD (C vs R)	0.00 (–0.02, +0.02)	0.02 (–0.08, +0.13)	0.00 (–0.02, +0.02)	0.00 (–0.02, +0.02)	0.00 (–0.11, +0.11)	0.00 (–0.02, +0.02)
dLPOD (CP vs CC)	0.00 (–0.02, +0.02)	0.01 (–0.10, +0.12)	0.00 (–0.02, +0.02)	0.00 (–0.02, +0.02)	0.01 (–0.10, +0.12)	0.00 (–0.02, +0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

DNA, which is stable and unaffected by growth environment. The fragment is a genetic sequence that is unique to the genus *Salmonella*, thus providing a highly reliable indicator that the organism is present. The BAX System simplifies the PCR process by combining the requisite primers, polymerase, and nucleotides into a stable, dry, manufactured tablet already packaged inside the PCR tubes. After amplification, these tubes remain sealed for the detection phase, thus significantly reducing the potential for contamination with one or more molecules of amplified PCR product.

This automated BAX System method uses fluorescent detection to analyze PCR product. One PCR primer for each target (one *Salmonella*-specific target and an internal control) contains a fluorescent dye (two different dyes, one for each target) as a constituent of the primer as well as a quencher (the unimolecular combination of a primer, fluorescent dye, and quencher constitute a Scorpion™ Probe). When incorporated into a PCR product, the dye and quencher are spatially separated, which causes an increase in emission signal. The BAX System measures the magnitude and characteristics of fluorescent signal change. An analysis by the BAX System software algorithm then evaluates that data to determine a positive or negative result which is displayed as described below.

B. Apparatus and Reagents

Items (**a**)–(**h**) are part of the DuPont BAX System Start-Up Package available from DuPont Nutrition & Health (Wilmington, DE; www.fooddiagnostics.dupont.com).

Items (**i**)–(**l**) are part of the DuPont BAX System Real-Time PCR Assay for *Salmonella* available from DuPont Nutrition & Health (Cat. No. D14306040).

(**a**) *DuPont BAX System Q7 cycler/detector with computer workstation.*

(**b**) *DuPont BAX System application software.*

(**c**) *Cluster tubes with caps and racks.*—For lysis.

(**d**) *Capping/decapping tools.*—For removing and sealing cluster tube caps and PCR tube caps without jarring the contents.

(**e**) *Heating and cooling blocks with inserts.*—For maintaining lysis tubes at 37 ± 2, 95 ± 2, and 4°C. [*Note*: The DuPont Thermal Block (Cat. No. D14614252) may also be used to maintain appropriate temperatures for lysis tubes.]

(**f**) *Pipets.*—For transferring reagents; two adjustable mechanical pipets covering 20–200 and 5–50 µL; one repeating pipet; and one multichannel pipet covering eight channels and 550 µL. Pipets should be calibrated to deliver required volumes within 10%.

(**g**) *Pipet tips with barriers.*—0.5–250 µL, 0.5–100 µL extended barrier; 5 mL repeater pipet tips.

(**h**) *PCR tube holders.*—For transferring a rack of tubes from the cooling block to the cycler/detector.

(**i**) *PCR tubes with tablets.*

(**j**) *Flat optical caps for PCR tubes.*

(**k**) *Lysis buffer.*

(**l**) *Protease.*

(**m**) *Incubators.*—For maintaining media at 35 ± 1 and 39–42°C.

(**n**) *Stomacher.*—Seward model 400 or equivalent for mixing the sponge sample with enrichment media.

(**o**) *Appropriate confirmatory media for culture confirmation.*—Rappaport-Vassiliadis Soya Peptone (RVS), Selenite Cystine (SC), tetrathionate-Hajna (TT-Hajna) and tetrathionate (TT) broths, Xylose Lysine Desoxycholate (XLD), Xylose Lysine Tergitol 4 (XLT4), Hektoen Enteric (HE), Brilliant Green Sulfa (BGS), and Bismuth Sulfite (BS) agars.

C. Media

(**a**) *BAX System MP media.*—DuPont Cat. No. D12404925 (bulk powder) or D12745725 (StatMedia™ soluble packets).

(**b**) *Brain Heart Infusion (BHI) broth.*—Oxoid Cat. No. CM 1032 or equivalent.

(**c**) *Buffered Peptone Water (BPW).*—Oxoid Cat. No. CM 0509 or equivalent.

(**d**) *mTSB+n.*—Oxoid Cat. No. CM0989B or equivalent plus 2 mg/L novobiocin. Autoclave at 121°C for 15 min before addition of filter-sterilized novobiocin.

(**e**) *mTSB+caa+n.*—Oxoid Cat. No. CM0989B or equivalent plus 10 g/L casamino acids (casein acid hydrolysate) and 8 mg/L novobiocin. Autoclave at 121°C for 15 min before addition of filter-sterilized novobiocin.

(**f**) *Lactose broth (LB).*—Oxoid Cat. No. CM0137 or equivalent.

(**g**) *Brilliant green water.*—Prepare brilliant green water by adding 2 mL 1% brilliant green dye solution, **C**(**j**), per 1000 mL sterile distilled water. Let container stand undisturbed for 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, at 35°C for 24 ± 2 h.

(**h**) *Reconstituted nonfat dry milk.*—Suspend 100 g dehydrated nonfat dry milk in 1 L distilled water. Swirl until dissolved. Autoclave at 121°C for 15 min.

(**i**) *Universal preenrichment broth.*—Add 5 g tryptone, 5 g proteose peptone, 15 g potassium phosphate, 7 g sodium phosphate, 5 g sodium chloride, 0.5 g dextrose, 0.25 g magnesium sulfate, 0.1 g ferric ammonium citrate, and 0.2 g sodium pyruvate to 1 L distilled water. Heat ingredients with gentle agitation to dissolve, dispense, and autoclave at 121°C for 15 min. Final pH should be 6.3 ± 0.2.

(**j**) *1% Aqueous brilliant green dye solution.*—Dissolve 1 g dye in sterile water. Dilute to 100 mL.

(**k**) *Tryptic soy broth (TSB)*—Suspend 17 g tryptose, 3 g phytone, 5 g sodium chloride, 2.5 g potassium phosphate dibasic, and 2.5 g glucose in 1 L distilled water. Heat gently to dissolve, dispense into containers, and then autoclave 15 min at 121°C. Final pH is 7.3 ± 0.2.

D. Sample Enrichment

(**a**) *Ground beef, ground beef with soy, beef trim (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BPW, **C**(**c**). Incubate, **B**(**m**), at 35°C for 20–24 h.

(**b**) *Ground beef (375 g).*—Weigh 375 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 1500 mL prewarmed (45°C) mTSB+n, **C**(**d**). Incubate, **B**(**m**), at 39–42°C for 22–26 h.

(**c**) *Ground beef with soy (325 g).*—Weigh 325 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 975 mL prewarmed (35°C) mTSB+caa+n, **C**(**e**). Incubate, **B**(**m**), at 35°C for 20–24 h.

(**d**) *Beef trim (325 g).*—Weigh 325 g test portion into sterile container. Hand massage to homogenize sample for 2 min with

Figure 2013.02. Results are displayed on the computer screen after approximately 1 hr 10 min automated processing as a grid of icons representing the PCR outcome for each sample.

1500 mL prewarmed (41°C) BAX System MP media, **C**(**a**). Incubate, **B**(**m**), at 39–42°C for 16–24 h.

(**e**) *Frankfurters (325 g).*—Weigh 325 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 1400 mL prewarmed (35°C) BPW, **C**(**c**). Add additional BPW to reach a total media volume of 2925 mL. Incubate, **B**(**m**), at 35°C for 18–24 h.

(**f**) *Shrimp and peanut butter (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) LB, **C**(**f**). Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for peanut butter.

(**g**) *Ground turkey and chicken wings (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BPW, **C**(**c**). Incubate, **B**(**m**), at 35°C for 16–24 h.

(**h**) *Poultry rinse (30 mL).*—Combine 30 mL BPW rinsate with 30 mL prewarmed (35°C) BPW, **C**(**c**), into sterile container. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**i**) *Dried eggs (25 g).*—Weigh 25 g test portion into sterile container. Add approximately 15 mL prewarmed (35°C) LB, **C**(**f**), to sample and stir to smooth. Add three additional aliquots of LB of 10, 10, and 190 mL (total media volume 225 mL), stirring after each addition. Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**j**) *Dried eggs, ice cream, and peanut butter (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BPW, **C**(**c**). Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for peanut butter.

(**k**) *Shell eggs (approximately 1000 mL).*—Combine 20 eggs into sterile container with 2000 mL prewarmed (42°C) BAX System MP media, **C**(**a**). Incubate, **B**(**m**), at 42°C for 48 h.

(**l**) *Frozen peas, cream cheese, ice cream, and infant formula (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) LB, **C**(**f**). Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate at 35°C for 22–26 h.

(**m**) *Frozen peas (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BAX System MP media, **C**(**a**). Incubate, **B**(**m**), at 35°C for 22–26 h.

(**n**) *Cream cheese (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) BAX System MP media, **C**(**a**). Incubate, **B**(**m**), at 35°C for 12–24 h.

(**o**) *Fresh bagged lettuce (25 g).*—Weigh 25 g test portion into sterile container. Add 225 mL prewarmed (35°C) LB, **C**(**f**), and swirl 25 times clockwise and 25 times counterclockwise. Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**p**) *Fresh bagged lettuce (25 g).*—Weigh 25 g test portion into sterile container. Add 225 mL prewarmed (35°C) BAX System MP media, **C**(**a**), and swirl 25 times clockwise and 25 times counterclockwise. Incubate, **B**(**m**), at 35°C for 10–24 h.

(**q**) *Ice cream (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) brilliant green water, **C**(**g**). Incubate, **B**(**m**), at 35°C for 22–26 h.

(**r**) *Orange juice (25 mL).*—Weigh 25 g test portion into 225 mL prewarmed (35°C) universal preenrichment broth, **C**(**i**), and swirl thoroughly. Let stand at room temperature for 55–65 min. Do not mix or adjust pH. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for this sample type.

(**s**) *Orange juice (25 mL).*—Weigh 25 g test portion into 225 mL prewarmed (41°C) BAX System MP media, **C**(**a**), and swirl thoroughly. Incubate, **B**(**m**), at 39–42°C for 22–26 h.

Note: Regrowth is required for this sample type.

(**t**) *Nonfat dry milk (25 g).*—Pour 25 g sample slowly over the surface of 225 mL prewarmed (35°C) brilliant green water, **C**(**g**). Let stand at room temperature for 55–65 min. Do not mix or adjust pH. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for this sample type.

(**u**) *Stainless steel, ceramic tile, and plastic.*—Add 225 mL prewarmed (35°C) LB, **C**(**f**), to environmental sponge in sample bag and swirl thoroughly. Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**v**) *Stainless steel, ceramic tile, and plastic.*—Add 225 mL prewarmed (35°C) BPW, **C**(**c**), to environmental sponge in sample bag and swirl thoroughly. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 18–24 h.

(**w**) *Cocoa (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL reconstituted nonfat dry milk, **C**(**h**). Let stand at room temperature for 55–65 min, and then swirl thoroughly to mix. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Add 0.45 mL 1% aqueous brilliant green dye solution, **C**(**j**), and mix well. Incubate, **B**(**m**), at 35°C for 22–26 h. Transfer 10 µL enrichment to 500 µL BHI broth, **C**(**b**), before processing. No additional incubation is required.

(**x**) *White pepper (25 g).*—Weigh 25 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with 225 mL prewarmed (35°C) TSB, **C**(**k**). Let stand at room temperature for 55–65 min. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

(**y**) *Dry pet food (375 g).*—Weigh 375 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample for 2 min with approximately one-third to one-half of 3375 mL prewarmed (35°C) LB, **C**(**f**). Add the remainder of the prewarmed media. Let stand at room temperature for 55–65 min, and then swirl thoroughly to mix. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for this sample type.

(**z**) *Dry pet food (375 g).*—Weigh 375 g test portion into sterile container. Use a stomacher, **B**(**n**), to homogenize sample

for 2 min with approximately one-third to one-half of 3375 mL prewarmed (35°C) BPW, **C**(**c**). Add the remainder of the prewarmed media. Adjust pH to 6.8 ± 0.2 using 1 N HCl or 1 N NaOH, if necessary. Incubate, **B**(**m**), at 35°C for 22–26 h.

Note: Regrowth is required for this sample type.

E. Regrowth

(**a**) After incubation, transfer 10 µL of the enrichment to 500 µL prewarmed (37°C) BHI broth, **C**(**b**). Incubate, **B**(**m**), at 37°C for 3 h.

(**b**) Regrowth is required for orange juice, nonfat dry milk, peanut butter, and dry pet food samples. For cocoa, a dilution without additional incubation is required. For all other matrixes, regrowth is either optional or not required.

F. Assay

(**a**) After enriching the sample, turn on the heating blocks, **B**(**e**), and set temperatures to 37 and 95°C. Make sure that the cooling blocks have been refrigerated overnight or otherwise chilled at 2–8°C.

(**b**) Create a rack file by following prompts in the Rack Wizard, **B**(**b**), to enter identifying data on the entire rack and on the individual samples.

(**c**) Label and arrange cluster tubes, **B**(**c**), in the cluster tube rack, according to the rack file.

(**d**) Prepare the lysis reagent by adding 150 µL protease, **B**(**l**), to one 12 mL bottle lysis buffer, **B**(**k**). Transfer 200 µL prepared lysis reagent to each of the cluster tubes.

(**e**) Transfer 5 µL enriched sample to the corresponding cluster tubes. Secure caps with the capping/decapping tool, **B**(**d**).

(**f**) Heat cluster tubes at 37°C for 20 min.

(**g**) Heat cluster tubes at 95°C for 10 min.

(**h**) Cool cluster tubes at 2–8° for at least 5 min.

(**i**) Warm up the cycler/detector, **B**(**a**), by selecting RUN FULL PROCESS from the Operations menu of the application window, **B**(**b**).

(**j**) Place a PCR tube holder, **B**(**h**), on the PCR cooling block, **B**(**e**). Insert one PCR tube, **B**(**i**), per sample into the holder and remove caps with the capping/decapping tool, **B**(**d**).

(**k**) Using a multichannel pipet, **B**(**f**), transfer 30 µL of sample lysate to PCR tubes, **B**(**i**). Seal with flat optical caps, **B**(**j**), with the capping/decapping tool, **B**(**d**).

(**l**) Follow screen prompts, **B**(**b**), to load samples into the cycler/detector, **B**(**a**), and begin the program. At the completion of the PCR and detection process, follow the screen prompts to remove samples and display results.

G. Assay Results

The results are recorded on the rack display or from a spreadsheet printout of the results (called Detail View). Negative results are indicated by a green circle with (–) symbol, positive results are indicated by a red circle with (+) symbol, and indeterminate results are indicated with a yellow circle with (?) symbol. A yellow circle with a (?) symbol and a red slash indicate a low signal or signal error.

BAX System results are displayed as in Figure **2013.02**. figA

H. Confirmation

Presumptive positive results are confirmed by culture and the biochemical and serological protocols described in the appropriate reference method relevant to the matrix. For meat, poultry, and pasteurized egg products, follow the USDA-FSIS MLG Chapter 4 (http://www.fsis.usda.gov/wps/wcm/connect/700c05fe-06a2-492a-a6e1-3357f7701f52/MLG-4.pdf?MOD=AJPERES). For all other matrixes, follow the FDA-BAM Chapter 5 (http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070149.htm). Alternatively, matrixes may be confirmed as described in the Health Canada Compendium, Vol. 3, Laboratory Procedures for the Microbiological Examination of Foods, Health Canada, Health Products and Food Branch, where appropriate (http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/volume3-eng.php).

Results and Discussion

The results for orange juice are presented in Appendix 4, Tables 1–3. At each inoculation level, the BAX System method and the reference method demonstrated no significant statistical difference as indicated by POD analysis (the 95% confidence interval of the dLPOD included 0 in all cases). Two orange juice samples (one from each of two collaborator sites) returned a presumptive positive result with the test method but could not be culture confirmed. One sample indicated a very weak positive result, suggesting either a cross-contamination event (most likely during a sample transfer step) or a very low target cell density in the sample, which could be detected with the PCR method but was difficult to detect by culture. The second sample returned a strong positive result with the test method, so it is unclear what caused the discordant results between the test and reference methods. The remaining 502 orange juice samples tested from the alternative enrichment were in agreement with culture confirmation from the alternative enrichment broths.

The results for frankfurters are presented in Appendix 4, Tables 4–6. At each inoculation level, the BAX System method and the reference method demonstrated no significant statistical difference as indicated by POD analysis (the 95% confidence interval of the dLPOD included 0 in all cases). Two frankfurter samples, both from the same collaborator site, returned a presumptive positive result with the test method but could not be culture confirmed. Both samples indicated a very weak positive result, suggesting either a cross-contamination event or a very low target cell density in the sample, which could be detected with the PCR method but was difficult to detect by culture. The remaining 502 frankfurter samples analyzed with the alternative method were in agreement with culture confirmation results. One sample initially returned an indeterminate result with the test method and was retested according to the manufacturer's instructions. Upon retest, this sample returned a negative result, which was in agreement with culture confirmation results.

A POD summary of all test method results is shown in Table **2013.02**. Across all three inoculation levels for both matrixes, statistical analyses indicate that the test method presented demonstrates no significant differences from the reference methods. The within-laboratory component (S_r) of the reproducibility S_R value represents the sampling variability at very low spiking levels. It accounted for all of the S_R value

observed for each matrix collaboratively studied, the S_L value (between-laboratory effect components of S_R) being zero in both data sets at each partial response spike level. This acceptable interlaboratory reproducibility is supported by the insignificant homogeneity test P_T values (>0.1), which suggest that the laboratory POD values are not significantly different when allowance is made for the sampling variability. While interpretation of this latter test is subject to the study design, 10 or more laboratories with 12 replicate sample portions per level for each of three levels (high, low, and unspiked) per laboratory is deemed adequate for such studies.

The graphical representation of the data (Appendix 4, Figure 1) demonstrates that the dose-response curve for each matrix encompasses the partial response region required for qualitative detection method analysis. The 95% confidence interval of each dPOD value determined at each concentration contains zero, which is indicative of no significant difference between the candidate and reference methods and between the candidate presumptive result and candidate confirmed result.

Conclusions

Within the statistical constraints of these studies, no differences were found between the reference culture-based methods and the alternative BAX System method. These results indicate that the alternative method can be used to allow uncontaminated food to be released rapidly from a manufacturer's control and prevents *Salmonella*-contaminated foods from entering commerce. Furthermore, this test method can be a valuable tool for outbreak investigations when food contamination events occur.

Collaborator feedback indicated that the method was easy to use and that the clear yes/no results provided by the BAX System software were appreciated. Time and labor savings were cited as key advantages of the test method over the reference culture methods. No negative feedback regarding the method was provided by any of the collaborators.

The DuPont BAX System Real-Time PCR Assay for *Salmonella* was adopted as Official First Action status for the detection of *Salmonella* in a variety of foods, including raw ground beef, ground beef with soy, beef trim, frankfurters, shrimp, ground turkey, chicken wings, poultry rinse, dried eggs, shell eggs, fresh bagged lettuce, frozen peas, orange juice, cream cheese, nonfat dry milk, ice cream, peanut butter, cocoa, white pepper, infant formula, and dry pet food, and on stainless steel, ceramic tile, and plastic surfaces.

Acknowledgments

We sincerely thank the following collaborators for their dedicated participation in this study:

Bryan Dieckelman, Deibel Laboratories (Lincolnwood, IL)

Tom Donohue, LeAnne Hahn, and Catherine Churchill, Deibel Laboratories (Madison, WI)

Hesham Elgaali, Indiana Department of Health (Indianapolis, IN)

Willis Fedio, New Mexico State University (Las Cruces, NM)

Elizabeth Galbraith, DuPont Nutrition & Health (Waukesha, WI)

Brian Kupski, Silliker Laboratories (Crete, IL)

Kristina McCallum and Dominika Kondratko, Colorado Department of Agriculture (Denver, CO)

Guy McWhorter, ABC Research Laboratories (Gainesville, FL)

Joe Meyer and Demi Swift, Covance Laboratories (Monona, WI)

Roy Radcliff and Dorn Clark, Jr, Marshfield Food Safety (Marshfield, WI)

Dora Rodgers, Samantha Scott, and Joseph Putrow, Wisconsin Department of Agriculture (Madison, WI)

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We would also like to thank Nicolette Blubaugh (DuPont Nutrition & Health, Wilmington, DE) for invaluable assistance in the preparation of this manuscript and Maria Nelson (AOAC INTERNATIONAL, Gaithersburg, MD) for her expertise and assistance in the design and execution of this study.

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FOOD BIOLOGICAL CONTAMINANTS

Evaluation of 3M™ Molecular Detection Assay (MDA) *Salmonella* for the Detection of *Salmonella* in Selected Foods: Collaborative Study

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The 3M™ Molecular Detection Assay (MDA) *Salmonella* is used with the 3M™ Molecular Detection System for the detection of *Salmonella* spp. in food, food-related, and environmental samples after enrichment. The assay utilizes loop-mediated isothermal amplification to rapidly amplify *Salmonella* target DNA with high specificity and sensitivity, combined with bioluminescence to detect the amplification. The 3M MDA *Salmonella* method was compared using an unpaired study design in a multilaboratory collaborative study to the U.S. Department of Agriculture/Food Safety and Inspection Service-*Microbiology Laboratory Guidebook* (USDA/FSIS-MLG 4.05), *Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg and Catfish Products* for raw ground beef and the U.S. Food and Drug Administration/*Bacteriological Analytical Manual* (FDA/BAM) Chapter 5 *Salmonella* reference method for wet dog food following the current AOAC guidelines. A total of 20 laboratories participated. For the 3M MDA *Salmonella* method, raw ground beef was analyzed using 25 g test portions, and wet dog food was analyzed using 375 g test portions. For the reference methods, 25 g test portions of each matrix were analyzed. Each matrix was artificially contaminated with *Salmonella* at three inoculation levels: an uninoculated control level (0 CFU/test portion), a low inoculum level (0.2–2 CFU/test portion), and a high inoculum level (2–5 CFU/test portion). In this study, 1512 unpaired replicate samples were analyzed. Statistical analysis was conducted according to the probability of detection (POD). For the low-level raw ground beef test portions, the following dLPOD (difference between the POD of the reference and candidate method) values with 95% confidence intervals were obtained: –0.01 (–0.14, +0.12). For the low-level wet dog food test portions, the following dLPOD with 95% confidence intervals were obtained: –0.04 (–0.16, +0.09). No significant differences were observed in the number of positive samples detected by the 3M MDA *Salmonella* method versus either the USDA/FSIS-MLG or FDA/BAM methods.

For over 100 years, *Salmonella*, one of the most frequently reported causes of foodborne outbreaks, has been known to cause foodborne illness in humans (1). The bacterium has been implicated in outbreaks from a variety of foods including raw animal products, such as meat, poultry, eggs, dairy products, seafood, and some fruits and vegetables (2). In order to reduce outbreaks of Salmonellosis, a comprehensive farm-to-fork approach is needed. The detection of *Salmonella* can often be very time-consuming and expensive, as the presence of the microorganism in food usually does not affect the taste, smell, or appearance (3). The 3M™ Molecular Detection Assay (MDA) *Salmonella* method, in conjunction with 3M Buffered Peptone Water ISO (BPW ISO; 4), uses a combination of loop-mediated isothermal DNA amplification and bioluminescence detection to detect *Salmonella* in enriched food, feed, and environmental samples.

The 3M MDA *Salmonella* method allows for next-day detection of *Salmonella* species. After 18–24 h of enrichment using prewarmed (37 ± 1°C) 3M BPW ISO medium, *Salmonella* detection is performed by the 3M MDA *Salmonella* method. Presumptive positive results are reported in real time; negative results are displayed after completion of the assay.

Prior to the collaborative study, the 3M MDA *Salmonella* method was certified as a *Performance Tested Method* (PTM) following the AOAC guidelines for harmonized PTM studies (5). The aim of the PTM study was to demonstrate that the 3M MDA *Salmonella* method could detect *Salmonella* in selected foods as claimed by the manufacturer. For the 3M MDA *Salmonella* evaluation, six matrices were analyzed: raw ground beef (25 g), processed breaded chicken (325 g), liquid egg (100 g), shrimp (25 g), fresh spinach (25 g), and wet dog food (375 g). All other

Submitted for publication June 28, 2013.
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Appendixes are available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI: 10.5740/jaoacint.13-227

PTM parameters (inclusivity, exclusivity, ruggedness, stability, and lot-to-lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method was awarded PTM certification number 031208 on March 30, 2012.

The aim of this collaborative study was to compare the 3M MDA *Salmonella* method to the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS)-*Microbiology Laboratory Guidebook* (MLG) 4.05 (6) for raw ground beef and the U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) Chapter 5 (7) method for wet dog food.

Collaborative Study

Study Design

For this collaborative study, two matrices, raw ground beef (80% lean) and wet dog food (canned beef chunks), were analyzed. The matrices were obtained from local retailers and screened for the absence of *Salmonella* by preparing one bulk sample and analyzing five sample replicates (25 g) by the appropriate reference method. The screening indicated an absence of the target organism. The raw ground beef was artificially contaminated with *Salmonella* Ohio Sequence Types (STS) 81 and the wet dog food with *Salmonella* Poona National Collection of Type Cultures (NCTC) 4840. There were two inoculation levels for each matrix: a high inoculation level of approximately 2–5 CFU/test portion and a low inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions was also included for each matrix at 0 CFU/test portion.

Twelve replicate samples from each of the three contamination levels of product were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by the 3M MDA *Salmonella* method and either the USDA/FSIS-MLG (raw ground beef) or FDA/BAM (wet pet food) reference method due to different sample enrichments for the candidate method and the reference methods. For both matrices, collaborators were sent an additional 30 g test portion and instructed to conduct a total aerobic plate count (APC) following the FDA/BAM Chapter 3 on the day samples were received to determine the total aerobic microbial load.

A detailed collaborative study packet outlining all necessary information related to the study including media preparation, method-specific test portion preparation, and documentation of results was sent to each collaborating laboratory prior to the initiation of the study.

Preparation of Inocula and Test Portions

The *Salmonella* cultures used in this evaluation were propagated in 10 mL of Brain Heart Infusion broth from a Q Laboratories frozen stock culture held at –70°C. The broth was incubated for 18–24 h at 35 ± 1°C. Appropriate dilutions were prepared based on previously established growth curves for both low and high inoculation levels, resulting in fractional positive outcomes for at least one level. For both test portion sizes, a bulk lot of each matrix was inoculated with a liquid inoculum and mixed thoroughly by hand-kneading to ensure an even distribution of microorganisms. The matrices were inoculated on the day of shipment so that all test portions would be held for 96 h before testing was initiated. For analysis of the raw ground beef, the bulk lot of test material was divided into 30 g portions for shipment to the collaborators. For analysis of the wet dog food, 25 g of inoculated test product was mixed with 350 g of uninoculated test product for shipment to the collaborators for analysis by the 3M MDA *Salmonella* method. For analysis by the reference method, collaborators received 30 g portions.

To determine the level of *Salmonella* spp. in the matrices, a five-tube most probable number (MPN) was conducted by the coordinating laboratory on the day of initiation of analysis using the FDA/BAM Chapter 5 reference method for wet pet food or the USDA/FSIS-MLG 4.05 reference method for raw ground beef. From both the high and low inoculated levels, five 100 g test portions, the reference method test portions, and five 10 g test portions were analyzed using the appropriate reference method enrichment broth. The MPN and 95% confidence intervals were calculated from the high, low, and uninoculated levels using the MPN Calculator (www.lcfltd.com/customer/LCFMPNCalculator.exe; 8). Confirmation of the samples was conducted according to either the USDA/FSIS-MLG 4.05 or FDA/BAM Chapter 5 reference method, dependent on the matrix.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded three-digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transport Association. All samples were packed with cold packs to target a temperature of <7°C during shipment. Upon receipt, samples were held by the collaborating laboratory at refrigerated temperature (3–5°C) until the following Monday, when analysis was initiated. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as "temperature control." Participants were instructed to record the temperature of this portion upon receipt of the shipment, document the results on the Sample Receipt Confirmation form provided, and fax to the Study Director.

Additional shipments of raw ground beef test portions were made by the sponsoring laboratory when aberrant results were observed. Further investigation of the results indicated that each participating collaborator detected the presence of the target analyte in the uninoculated control samples sent in the first shipment. In each case, the same species was reported for the control samples, which may have been due to cross-contamination. As a result, new test portions of raw ground beef were shipped and analyzed by each of the collaborating laboratories.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method for each matrix. For both matrices, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls for each method). For the analysis of the raw ground beef test portions by the 3M MDA *Salmonella* method, a 25 g portion was enriched with 225 mL of prewarmed (37 ± 1°C) 3M BPW

ISO, homogenized for 2 min and incubated for 18 h at 37 ±1°C. For the wet dog food test portions analyzed by the 3M MDA *Salmonella* method, a 375 g portion was enriched with 3375 mL prewarmed (37 ± 1°C) 3M BPW ISO, homogenized for 2 min and incubated for 18 h at 37 ± 1°C.

Following enrichment, samples were assayed by the 3M MDA *Salmonella* method and confirmed following the standard reference method. Both test portion sizes analyzed by the 3M MDA *Salmonella* method were compared to samples (25 g) analyzed using either the USDA/FSIS-MLG or FDA/BAM reference method in an unpaired study design. All positive test portions were biochemically confirmed by the API 20E biochemical test, AOAC *Official Method* **978.24**, or by the VITEK 2 GN identification test, AOAC *Official Method* **2011.17**. Serological testing was also performed.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and the 3M MDA *Salmonella* method on the data sheets provided. The data sheets were submitted to the Study Director at the end of each week of testing for analysis. The results of each test portion for each sample were compiled by the Study Director and the qualitative 3M MDA *Salmonella* results were compared to the reference method for statistical analysis. Data for each test portion size were analyzed using the probability of detection (POD; 9). If the confidence interval of a dLPOD did not contain zero, then that would indicate a statistically significant difference between the candidate method and the reference method at the 5% confidence level (9).

AOAC Official Method 2013.09
Salmonella in Selected Foods
3M™ Molecular Detection Assay (MDA) *Salmonella* Method
First Action 2013

[Applicable to detection of *Salmonella* in raw ground beef (25 g), processed breaded chicken (325 g), liquid egg (100 g), shrimp (25 g), fresh spinach (25 g), and wet dog food (375 g)].

See Tables **2013.09A** and **B** for a summary of results of the inter-laboratory study.

See Appendix Tables A and B for detailed results of the inter-laboratory study.

A. Principle

The 3M Molecular Detection Assay (MDA) *Salmonella* method is intended for use with the 3M Molecular Detection System for the rapid and specific detection of *Salmonella* spp. in food, feed, and environmental samples after enrichment. After enrichment in prewarmed 3M Buffered Peptone Water ISO (3M BPW ISO) medium, the 3M MDA *Salmonella* test utilizes loop-mediated isothermal amplification to rapidly amplify *Salmonella* target DNA with high specificity and sensitivity, combined with bioluminescence to detect the amplification. Presumptive positive results are reported in real time; negative results are displayed after the assay is completed.

B. Apparatus and Reagents

Items (**b**)–(**g**) are available as the 3M MDA *Salmonella* kit from 3M Food Safety (St. Paul, MN).

(**a**) *3M Molecular Detection System*.—Available from 3M Food Safety.

(**b**) *3M MDA Salmonella reagent tubes*.—12 strips of eight tubes.

(**c**) *Lysis solution (LS) tubes*.—12 strips of eight tubes.

(**d**) *Extra caps*.—12 strips of eight caps.

(**e**) *Negative control (NC)*.—One vial (2 mL).

(**f**) *Reagent control (RC)*.—Eight reagent tubes.

(**g**) *Quick start guide.*

(**h**) *3M Molecular Detection Speed Loader Tray*.—Available from 3M Food Safety.

(**i**) *3M Molecular Detection Chill Block Tray and Chill Block Insert*.—Available from 3M Food Safety.

(**j**) *3M Molecular Detection Heat Block Insert*.—Available from 3M Food Safety.

(**k**) *3M Molecular Detection Cap/Decap Tool for reagent tubes*.—Available from 3M Food Safety.

(**l**) *3M Molecular Detection Cap/Decap Tool for lysis tubes*.—Available from 3M Food Safety.

(**m**) *Empty lysis tube rack*.—Available from 3M Food Safety.

(**n**) *Empty reagent tube rack*.—Available from 3M Food Safety.

(**o**) *3M BPW ISO*.—Available from 3M Food Safety. Formulation equivalent to ISO 6579:2002 Annex B (4).

(**p**) *Disposable pipet*.—Capable of 20 µL.

(**q**) *Multichannel (eight-channel) pipet.*—Capable of 20 µL.

(**r**) *Sterile filter tip pipet tips*.—Capable of 20 µL.

(**s**) *Filter stomacher bags*.—Seward Laboratory Systems Inc., Bohemia, NY, or equivalent.

(**t**) *Stomacher*.—Seward Laboratory Systems Inc. or equivalent.

(**u**) *Thermometer*.—Calibrated range to include 100 ± 1°C.

(**v**) *Dry double block heater unit or water bath*.—Capable of maintaining 100 ± 1°C.

(**w**) *Incubators*.—Capable of maintaining 37 ± 1°C.

(**x**) *Freezer*.—Capable of maintaining –10 to –20°C, for storing the 3M Molecular Detection Chill Block Tray.

(**y**) *Refrigerator*.—Capable of maintaining 2–8°C, for storing the 3M MDA.

(**z**) *Computer*.—Compatible with the 3M Molecular Detection Instrument.

C. General Instructions

(**a**) Store the 3M MDA *Salmonella* kit at 2–8°C. Do not freeze. Keep kit away from light during storage. After opening the kit, check that the foil pouch is undamaged. If the pouch is damaged, do not use. After opening, unused reagent tubes should always be stored in the resealable pouch with the desiccant inside to maintain stability of the lyophilized reagents. Store resealed pouches at 2–8°C for no longer than 60 days. Do not use 3M MDA *Salmonella* past the expiration date.

(**b**) The 3M Molecular Detection Instrument is intended for use with samples that have undergone heat treatment during the assay lysis step, which is designed to destroy organisms present in the sample. Samples that have not been properly heat-treated during the assay lysis step may be considered a potential

Table 2013.09A. POD summary of raw ground beef (25 g) results for the 3M MDA *Salmonella* method[a]

	Inoculation level		
	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	1/120	69/120	120/120
Candidate presumptive (CP) POD	0.01 (0.00, +0.05)	0.58 (+0.48, +0.67)	1.00 (+0.97, +1.00)
s_r[b]	0.09 (+0.08, +0.17)	0.51 (+0.45, +0.52)	0.00 (0.00, +0.18)
s_L[c]	0.00 (0.00, +0.04)	0.00 (0.00, +0.14)	0.00 (0.00, +0.18)
s_R[d]	0.09 (+0.08, +0.10)	0.51 (+0.45, +0.52)	0.00 (0.00, +0.24)
Candidate confirmed positive/total No. of samples analyzed	0/120	67/120	120/120
Candidate confirmed (CC) POD	0.00 (0.00, +0.03)	0.56 (+0.47, +0.65)	1.00 (+0.97, +1.00)
s_r[b]	0.00 (0.00, +0.17)	0.51 (+0.45, +0.52)	0.00 (0.00, +0.18)
s_L[c]	0.00 (0.00, +0.17)	0.00 (0.00, +0.11)	0.00 (0.00, +0.18)
s_R[d]	0.00 (0.00, +0.24)	0.51 (+0.46, +0.52)	0.00 (0.00, +0.24)
Positive reference samples/total No. of samples analyzed	0/120	68/120	119/120
Reference POD	0.00 (0.00, +0.03)	0.57 (+0.48, +0.66)	0.99 (+0.95, +1.00)
s_r[b]	0.00 (0.00, +0.17)	0.50 (+0.45, +0.52)	0.09 (+0.08, +0.17)
s_L[c]	0.00 (0.00, +0.17)	0.00 (0.00, +0.18)	0.00 (0.00, +0.04)
s_R[d]	0.00 (0.00, +0.24)	0.51 (+0.45, +0.52)	0.09 (+0.08, –0.11)
dLPOD (Candidate vs Reference)	0.00 (–0.03, +0.03)	–0.01 (–0.14, +0.12)	0.01 (–0.02, +0.05)
dLPOD (CP vs CC)	0.01 (–0.02, +0.05)	0.02 (–0.11, +0.15)	0.00 (–0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability SD.

[c] Among-laboratory SD.

[d] Reproducibility SD.

biohazard and should not be inserted into the 3M Molecular Detection Instrument.

(**c**) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

(**d**) After use, the enrichment medium and the 3M MDA *Salmonella* tubes can potentially contain pathogenic materials. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal.

Periodically decontaminate laboratory benches and equipment (pipets, cap/decap tools, etc.) with a 1–5% (v/v in water) household bleach solution or DNA removal solution.

D. Sample Enrichment

Prewarm 3M BPW ISO enrichment medium to 37 ± 1°C.

Aseptically combine the enrichment medium and sample following the outline in Table **2013.09C**. For all meat and highly particulate samples, the use of filter bags is recommended. Homogenize thoroughly for 2 min. Incubate at 37 ± 1°C.

E. Preparation of the 3M Molecular Detection Speed Loader Tray

Wet a cloth or paper towel with a 1–5% (v/v in water) household bleach solution and wipe the 3M Molecular Detection Speed Loader Tray. Rinse the tray with water. Use a disposable towel to wipe the tray dry. Ensure the 3M Molecular Detection Speed Loader Tray is dry before use.

F. Preparation of the 3M Molecular Detection Chill Block Insert

Before using the 3M Molecular Detection Chill Block Insert, ensure it has been stored on the 3M Molecular Detection Chill Block Tray in the freezer (–10 to –20°C) for a minimum of 2 h before use. When removing the 3M Molecular Detection Chill Block Insert from the freezer for use, remove it and the 3M Molecular Detection Chill Block Tray together. Use the insert and tray within 20 min.

G. Preparation of the 3M Molecular Detection Heat Block Insert

Place the 3M Molecular Detection Heat Block Insert in a dry double block heater unit. Turn on the dry block heater unit and set the temperature to allow the 3M Molecular Detection Heat Block Insert to reach and maintain a temperature of 100 ± 1°C.

Note: Depending on the heater unit, allow approximately 30–50 min for the 3M Molecular Detection Heat Block Insert to reach temperature. Using a calibrated thermometer, verify that the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C.

Table 2013.09B. POD Summary of wet pet food (375 g) results for the 3M MDA *Salmonella* method[a]

	Inoculation level		
	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	1/132	65/132	131/132
Candidate presumptive (CP) POD	0.01 (0.00, +0.04)	0.49 (+0.40, +0.58)	0.99 (+0.96, +1.00)
s_r[b]	0.09 (+0.08, +0.16)	0.51 (+0.46, +0.52)	0.09 (+0.08, +0.16)
s_L[c]	0.00 (0.00, +0.04)	0.00 (0.00, +0.14)	0.00 (0.00, +0.04)
s_R[d]	0.09 (+0.08, +0.10)	0.51 (+0.46, +0.52)	0.09 (+0.08, +0.10)
Candidate confirmed positive/total No. of samples analyzed	0/132	65/132	131/132
Candidate confirmed (CC) POD	0.00 (0.00, +0.03)	0.49 (+0.40, +0.58)	0.99 (+0.96, +1.00)
s_r[b]	0.00 (0.00, +0.17)	0.51 (+0.46, +0.52)	0.09 (+0.08, +0.16)
s_L[c]	0.00 (0.00, +0.17)	0.00 (0.00, +0.14)	0.00 (0.00, +0.04)
s_R[d]	0.00 (0.00, +0.23)	0.51 (+0.46, +0.52)	0.09 (+0.08, +0.10)
Positive reference samples/total No. of samples analyzed	0/132	70/132	132/132
Reference POD	0.00 (0.00, +0.03)	0.53 (+0.44, +0.62)	1.00 (+0.97, +1.00)
s_r[b]	0.00 (0.00, +0.17)	0.52 (+0.46, +0.52)	0.00 (0.00, +0.17)
s_L[c]	0.00 (0.00, +0.17)	0.00 (0.00, +0.09)	0.00 (0.00, +0.17)
s_R[d]	0.00 (0.00, +0.23)	0.52 (+0.47, +0.52)	0.00 (0.00, +0.23)
dLPOD (Candidate vs Reference)	0.00 (–0.03, +0.03)	–0.04 (–0.16, +0.09)	–0.01 (–0.04, +0.02)
dLPOD (CP vs CC)	0.01 (–0.02, +0.05)	0.00 (–0.13, +0.13)	0.00 (–0.03, +0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability SD.

[c] Among-laboratory SD.

[d] Reproducibility SD.

H. Preparation of the 3M Molecular Detection Instrument

Launch the 3M Molecular Detection Software and log in. Turn on the 3M Molecular Detection Instrument. Create or edit a run with data for each sample. Refer to the 3M Molecular Detection System User Manual for details.

Note: The 3M Molecular Detection Instrument must reach and maintain a temperature of 60°C before a run can be started. This heating step takes approximately 20 min and is indicated by an orange light on the instrument's status bar. When the instrument is ready to start a run, the status bar will turn green.

I. Lysis

Allow the LS tubes to warm up to room temperature by setting the rack on the laboratory bench for 2 h. Alternatives to equilibrate the LS tubes to room temperature are to incubate the LS tubes in a 37 ± 1°C incubator for 1 h or at room temperature overnight (16–18 h). Remove the enrichment broth from the incubator and gently agitate the contents. One LS tube is required for each sample and the NC sample. LS tube strips can be cut to the desired number. Select the number of individual LS tubes or eight-tube strips needed. Place the LS tubes in an empty rack. To avoid cross-contamination, decap strip at a time and use a new pipet tip for each transfer step. Transfer the enriched samples to LS tubes as described below:

Note: Transfer each enriched sample into individual LS tube first. Transfer the NC last.

Use the 3M Molecular Detection Cap/Decap Tool-Lysis to decap one LS tube strip—one strip at a time. Set the tool with cap attached aside on a clean surface. Transfer 20 µL of sample into an LS tube. Repeat transfer until each individual sample has been added to a corresponding LS tube in the strip. Use the 3M Molecular Detection Cap/Decap Tool-Lysis to recap the LS tube strip. Use the rounded side of the tool to apply pressure in a back-and-forth motion to ensure that the cap is tightly applied. Repeat as needed for the number of samples to be tested.

When all samples have been transferred, transfer 20 µL of NC into a LS tube. Use the 3M Molecular Detection Cap/Decap Tool-Lysis tool to recap the LS tube. Cover the rack of LS tubes with the rack lid and firmly invert three to five times

Table 2013.09C Sample enrichment protocols

Sample matrix	Sample size, g	Enrichment broth volume, mL	Enrichment time, h
Raw ground beef (27% fat)	25	225	18–24
Raw shrimp	25	225	18–24
Bagged spinach	25	225	18–24
Pasteurized liquid whole egg	100	900	18–24
Cooked breaded chicken	325	2925	18–24
Wet pet food (dog–beef cuts in gravy, canned)	375	3375	18–24

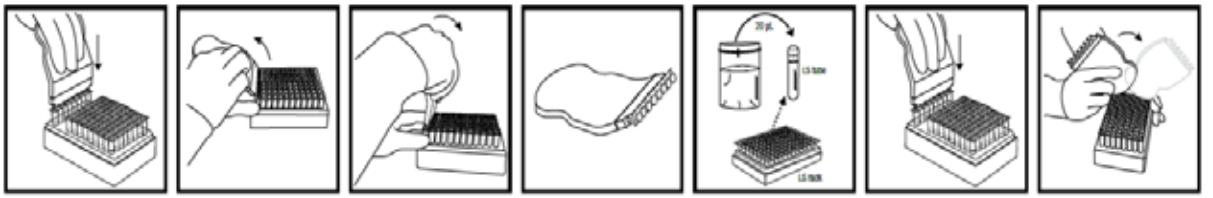

Figure 2013.09A. Transfer of enriched sample to Lysis Solution tube.

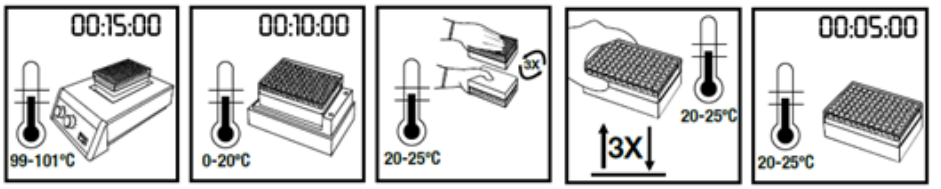

Figure 2013.09B. Sample Lysis.

to mix. Suspension has to flow freely inside the tube. *See* Figure **2013.09A**.

Verify that the temperature of the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C. Place the rack of LS tubes in the 3M Molecular Detection Heat Block Insert and heat for 15 ± 1 min. An alternative to using dry heat for the lysis step is to use a water bath at 100 ±1°C. Ensure that sufficient water is used to cover up to the liquid level in the LS tubes. Place the rack of LS tubes in the water bath at 100 ± 1°C and heat for 15 ± 1 min. Samples that have not been properly heat-treated during the assay lysis step may be considered a potential biohazard and should not be inserted into the 3M Molecular Detection Instrument.

Remove the rack of LS tubes from the heating block and allow to cool in the 3M Molecular Detection Chill Block Insert for 10 ± 1 min. Remove the rack lid during incubation on the 3M Molecular Detection Chill Block Insert. The LS solution may freeze when processing less than 48 LS tubes. Freezing of the LS solution will not affect your test. If freezing is observed, allow the LS tubes to thaw for 5 min before mixing.

Remove the rack of LS tubes from the 3M Molecular Detection Chill Block Insert/3M Molecular Detection Chill Block Tray system. Replace the lid on the rack of LS tubes and firmly invert three to five times to mix. Suspension has to flow freely inside the tube. Firmly tap the lysis tubes rack on the laboratory bench three to five times. Place the rack on the laboratory bench. Let it sit undisturbed for at least 5 min to allow the resin to settle. Do not mix or disturb the resin at the bottom of the tube. *See* Figure **2013.09B**.

J. Amplification

One reagent tube is required for each sample and the NC. Reagent tube strips can be cut to desired tube number. Select the number of individual reagent tubes or eight-tube strips needed. Place reagent tubes in an empty rack. Avoid disturbing the reagent pellets from the bottom of the tubes.

Select one RC tube and place in rack. To avoid cross-contamination, decap one reagent tubes strip at a time and use a new pipet tip for each transfer step. Transfer lysate to reagent tubes and RC tube as follows:

Transfer each sample lysate into individual reagent tubes first followed by the NC. Hydrate the RC tube last.

Warning: Care must be taken when pipetting LS, as carry-over of the resin may interfere with amplification.

(*1*) Use the 3M Molecular Detection Cap/Decap Tool-Reagent to decap the reagent tubes–one strip at a time. Discard cap. (*2*) Transfer 20 µL of sample lysate from the upper portion of the fluid in the LS tube into corresponding reagent tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times. (*3*) Repeat until individual sample lysate has been added to a corresponding reagent tube in the strip. (*4*) Cover the reagent tubes with the provided extra cap and use the rounded side of the 3M Molecular Detection Cap/Decap Tool-Reagent to apply pressure in a back-and-forth motion, ensuring that the cap is tightly applied. Repeat steps (*1*) to (*4*) as needed for the number of samples to be tested. When all sample lysates have been transferred, repeat steps (*1*) to (*4*) to transfer 20 µL of NC lysate into a reagent tube. Transfer 20 µL of NC lysate into a RC tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down five times. Load capped tubes into a clean and decontaminated 3M Molecular Detection Speed Loader Tray. Close and latch the 3M Molecular Detection Speed Loader Tray lid. *See* Figure **2013.09C**.

Review and confirm the configured run in the 3M Molecular Detection Software. Click the start button in the software and select instrument for use. The selected instrument's lid automatically opens. Place the 3M Molecular Detection Speed Loader Tray into the 3M Molecular Detection Instrument and close the lid to start the assay. Results are provided within 75 min, although positives may be detected sooner.

After the assay is complete, remove the 3M Molecular Detection Speed Loader Tray from the 3M Molecular Detection

Figure 2013.09C. Transfer of lysate to reagent tube.

Instrument and dispose of the tubes by soaking in a 1–5% (v/v in water) household bleach solution for 1 h and away from the assay preparation area.

Notice: To minimize the risk of false positives due to cross-contamination, never open reagent tubes containing amplified DNA. This includes RC, reagent, and matrix control tubes. Always dispose of sealed reagent tubes by soaking in a 1–5% (v/v in water) household bleach solution for 1 h away from the assay preparation area.

K. Results and Interpretation

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A positive or negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real time; negative and inspect results will be displayed after the run is completed. Presumptive positive results should be confirmed using your preferred method or as specified by the FDA/BAM (http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm070149.htm) or the USDA/FSIS-MLG (http://www.fsis.usda.gov/PDF/MLG_4_05.pdf; 6, 7), starting from the 3M BPW ISO, followed by secondary enrichment, plating, and confirmation of isolates using appropriate biochemical and serological methods.

Note: Even a negative sample will not give a zero reading as the system and 3M MDA *Salmonella* amplification reagents have a "background" relative light unit.

In the rare event of any unusual light output, the algorithm labels this as "inspect." 3M recommends the user to repeat the assay for any inspect samples. If the result continues to be inspect, proceed to confirmation test using your preferred method or as specified by local regulations.

Results

In this collaborative study, the 3M MDA *Salmonella* method was compared to the to the USDA/FSIS-MLG 4.05 reference method for raw ground beef and to the FDA/BAM, Chapter 5 reference method for wet dog food. A total of 20 laboratories throughout the United States participated in this study, with 14 laboratories submitting data for the raw ground beef and 16 laboratories submitting data for the wet dog food, as presented in Table 1. Each laboratory analyzed 36 test portions for each method: 12 inoculated with a high level of *Salmonella*, 12 inoculated with a low level of *Salmonella*, and 12 uninoculated controls. For each matrix, the actual level of *Salmonella* was determined by MPN determination on the day of initiation of analysis by the coordinating laboratory. The individual laboratory and sample results are presented in Tables 2 and 3. Tables **2013.09A** and **B** summarize the interlaboratory results for all foods tested, including POD statistical analysis (10). The results of the collaborating laboratories' APC analysis for each matrix are presented in Table C of the Appendix.

Raw Ground Beef (25 g Test Portions)

Raw ground beef test portions were inoculated at a low and high level and were analyzed (Table 2) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. The results presented for the raw ground beef were from a second shipment of test portions to the collaborating laboratories. The initial shipment of raw ground beef test portions sent to collaborators was discovered to contain contamination of the target analyte in the uninoculated control samples for each laboratory and therefore no data have been presented. Fourteen laboratories participated in the retest analysis of this matrix and the results of 10 laboratories were included in the statistical analysis. For the retest of the raw ground beef, laboratories 12, 16, 18, and 19 detected the presence of *Salmonella* spp. in either the candidate or reference method control replicates. Because of the potential for error, results from these laboratories were excluded from the statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.81 CFU/test portion (+0.62, +1.04) for the low level and 4.68 CFU/test portion (+3.22, +6.80) for the high level.

For the high level, 120 out of 120 test portions were reported as presumptive positive by the 3M MDA *Salmonella* method with all test portions confirming positive. For the low level, 67 out of 120 test portions were reported as presumptive positive by the 3M MDA *Salmonella* method with 65 test portions confirming positive. For the uninoculated controls, 1 out of 120 samples produced a presumptive positive result by the

Table 1. Participation of each collaborating laboratory[a]

Lab	Raw ground beef[b] (25 g test portions)	Wet dog food (375 g test portions)
1	Y	Y
2	Y	Y
3	N	Y
4	N	Y[c]
5	N	Y[c]
6	N	Y
7	N	Y
8	N	Y
9	Y	Y
10	Y	Y[c]
11	Y	Y
12	Y[c]	Y[c]
13	Y	Y
14	Y	Y
15	Y	Y
16	Y[c]	Y[c]
17	Y	N
18	Y[c]	N
19	Y[c]	N
20	Y	N

[a] Y = Collaborator analyzed the food type; N = collaborator did not analyze the food type.

[b] Data obtained from additional shipment of raw ground beef. Initial shipment of raw ground beef was not used for evaluation purposes and therefore the data has not been presented.

[c] Results were not used in statistical analysis due to laboratory error, or uninoculated control test portions were confirmed as *Salmonella*.

Table 2. Individual collaborator results for raw ground beef (25 g test portions)[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
	3M MDA *Salmonella*[b]																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–[c]	+	+	+	+	–	–	–[c]	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–[c]	–[c]	–[c]	+	+	+	–[c]	–	–	–	–[c]	–[c]	–[c]	–[c]	–	–[c]	–	–[c]	–	–[c]
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–[c]	–	–	–	–	–
16[d]	+	+	+	+	+	+	+	+	+	+	+	+	–[c]	–	+	+	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–[c]	–[c]	–[c]	–[c]	–	–
17	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
18[d]	+	+	+	+	+	+	+	+	+	+	+	+	–[c]	+	+	+	+	+	+	–[c]	+	+	–[c]	+	–[c]	+	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	–[c]	+
19[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	–	+	–[c]	–	–	–	–[c]	–[c]	–[c]	–[c]	–	–[c]	–[c]	–[c]	–[c]	–[c]
20	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
	USDA/FSIS-MLG[b]																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
12[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	+	–	–	–
13	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
16[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	+	–	–	–	–	–	–
17	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
18[d]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
19[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–
20	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Salmonella* spp. were detected in samples; – =*Salmonella* spp. were not detected in sample; NA = laboratory did not participate in this matrix, or results were not received.

[b] Sample results were obtained from the second shipment of raw ground beef test portions.

[c] Sample was presumptive positive on 3M MDA *Salmonella,* but confirmed negative, indicating a false-positive result.

[d] Results were not used in statistical analysis due to laboratory error.

Table 3. Individual collaborator results for wet dog food (375 g test portions)[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
3M MDA *Salmonella*																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–
5[b]	+	+	+	+	+	+	–	–	+	+	+	+	+	–	+	+	+	–	+	+	+	–	+	–	+	+	+	–	+	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–[c]	+	+	–[c]	–	+	+	+	–[c]	+	–	–	–[c]	–[c]	–	–	–	–[c]	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	–	–	+	+	–	–	–	–	–	–[c]	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
16[b]	+	+	+	+	–	–	+	+	+	+	+	+	+	+	–[c]	+	–	–	–	+	+	–	+	–	–	–[c]	–	+	–[c]	–[c]	+	+	+	–[c]	+	–
17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
FDA/BAM																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
4[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	+	–	–
5[b]	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
16[b]	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	–	–	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–
17	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
18	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
19	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
20	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

[a] + = *Salmonella* spp. were detected in samples; – =*Salmonella* spp. were not detected in sample; NA = laboratory did not participate in this matrix or results were not received.

[b] Results were not used in statistical analysis due to laboratory error.

[c] Sample was presumptive positive on 3M MDA *Salmonella,* but confirmed negative, indicating a false-positive result.

3M MDA *Salmonella* method with all test portions confirming negative. For test portions analyzed by the USDA/FSIS-MLG Method, 119 out of 120 high inoculum and 68 out of 120 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 120 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of –0.01 with 95% confidence intervals of (–0.14, +0.13) were obtained between the 3M MDA *Salmonella* method and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.02 with 95% confidence intervals of (–0.11, +0.15) was obtained between presumptive and confirmed 3M MDA *Salmonella* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, a $dLPOD_C$ value of 0.01 with 95% confidence intervals of (–0.02, +0.05) was obtained between the 3M MDA *Salmonella* method and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (–0.03, +0.03) was obtained between presumptive and confirmed 3M MDA *Salmonella* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.09A** and Figures 1A and B of the Appendix.

Wet Dog Food (375 g Test Portions)

Wet dog food test portions were inoculated at a low and high level and were analyzed (Table 3) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. Sixteen laboratories participated in the analysis of this matrix and the results of 11 laboratories were included in the statistical analysis. Laboratories 4, 5, 10, and 16 detected the presence of *Salmonella* spp. in either the candidate or reference method control replicates. Because of the potential for error, results from these laboratories were excluded from the statistical analysis. Laboratory 12 did not submit results due to cross-contamination of sample enrichments as reported by the analyst. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.72 CFU/test portion (+0.57, +0.90) for the low level and 5.34 CFU/test portion (+3.46, +8.24) for the high level.

For the high level, 131 out of 132 test portions were reported as presumptive positive by the 3M MDA *Salmonella* method with all test portions confirming positive. For the low level, 65 out of 132 test portions were reported as presumptive positive by the 3M MDA *Salmonella* method with all test portions confirming positive. For the uninoculated controls, 1 out of 132 samples produced a presumptive positive result by the 3M MDA *Salmonella* method with all test portions confirming negative. For test portions analyzed by the FDA/BAM method, 132 out of 132 high inoculum and 70 out of 132 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 132 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of –0.04 with 95% confidence intervals of (–0.16, +0.09) was obtained between the 3M MDA *Salmonella* method and the FDA/BAM method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (–0.13, +0.13) was obtained between presumptive and confirmed 3M MDA *Salmonella* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, a $dLPOD_C$ value of –0.01 with 95% confidence intervals of (–0.04, +0.02) was obtained between the 3M MDA *Salmonella* method and the FDA/BAM method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (–0.03, +0.03) was obtained between presumptive and confirmed 3M MDA *Salmonella* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.09B** and Figures 2A and B of the Appendix.

Discussion

For this collaborative study, samples were analyzed at both 25 and 375 g test portions as required by the current AOAC Guidelines (5), which require methods with more than one sample preparation or enrichment scheme to analyze one matrix per procedure. No negative feedback was provided by the collaborating laboratories in regard to the performance of the candidate method. Several collaborating laboratories expressed questions in regard to the AOAC study design of the collaborative study; others expressed concern with analyzing 375 g test portions. The concern with handling the larger test portions may have contributed to errors observed during testing that resulted in data not used in the statistical analysis.

During testing, four different laboratories detected the presence of *Salmonella* spp. in seven raw ground beef uninoculated control test portions. Additionally, four different laboratories detected the presence of *Salmonella* spp. in 15 wet pet food uninoculated control test portions. Due to detecting positive samples in the control test portions, the data provided by these laboratories were not included during the statistical analysis.

A root cause investigation to determine the source of contamination yielded the following possibilities: Due to the high number of samples analyzed, including test portions inoculated at a high inoculum level, contamination may have occurred during the transfer of enriched samples into the secondary selective enrichments or during the streaking of the reference agar plates. For the wet pet food, based on feedback from the collaborators, issues with storage during the incubation of the larger test portion sizes may have led to cross-contamination of the primary enrichments. Based on the fact that uninoculated control test portions were packaged 1 day prior to the inoculated test portions, contamination during test portion preparation at the coordinating laboratory is not believed to be the cause of the positive control samples.

During the analysis of both the raw ground beef and wet pet food, some laboratories produced false-positive results with the candidate method. The 3M Molecular Detection Assay is intended for use in a laboratory environment by professionals

trained in laboratory technique. Cross-contamination of samples resulting in false-positive results may occur if careful molecular techniques are not followed. To reduce the risk of cross-contamination, 3M recommends the use of sterile, aerosol barrier (filtered) molecular biology grade pipet tips. A new pipet tip should be used for each sample transfer, and the user may choose to add an intermediate transfer step in order to avoid pipet contamination, i.e., each enriched sample can be transferred into a sterile tube before proceeding to the lysis step. Discrepant results may be obtained if deviations from the method occur. Use of calibrated pipettors and thermometers is critical to ensure that correct volumes of samples, especially when hydrating the reagent tubes, and appropriate temperatures are utilized. It is recommended that users read and become familiar with the 3M MDA *Salmonella* product instructions and follow them carefully.

For either matrix, the collaborative study failed to show a statistically significant difference between the candidate method and the reference method using the POD model when the aforementioned four laboratories were removed from consideration.

Recommendations

It is recommended that the 3M MDA *Salmonella* method be adopted Official First Action for the detection of *Salmonella* in selected foods, including raw ground beef (25 g), processed breaded chicken (325 g), liquid egg (100 g), shrimp (25 g), fresh spinach (25 g), and wet dog food (375 g).

Acknowledgments

We extend our sincere thanks to the following collaborators for their dedicated participation in this study:

Joanne Ruebl, Cherney Microbiological Services, Ltd, Green Bay, WI

Jessica Dyszel and Mathew Vross, Richter International, Columbus, OH

Vikas Gill, U.S. FDA Center for Food Safety and Applied Nutrition, College Park, MD

Brad Stawick and Keith Blanchard, Microbac Laboratories, Inc., Warrendale, PA

Mark Horan and Delando Lewis, Microbac Laboratories, Inc., Baltimore, MD

Indaue Mello and Maria Ontiberos, Mars Petcare, US, Kansas City, MO

Jodene Jurgens and Leslie Thompson, Aegis, North Sioux City, SD

David Bosco, Food Safety Net Services, Fresno, CA

Amit Morey and Sergio Montez, Food Safety Net Services, San Antonio, TX

Kyle Newman, Venture Laboratories, Inc., Lexington, KY

Mary Bandu and Matt Oltman, Chestnut Laboratory, Springfield, MO

Robert Brooks, ATC Microbiology, LLC, North Little Rock, AR

Christine Gwinn and Scott Moosekian, Covance Laboratories, Inc., Battle Creek, MI

Joey Marchent, Gulf Coast Seafood Laboratory, FDA, Dauphin Island, AL

Kathleen T. Rajkowski, USDA Agricultural Research Services, Eastern Regional Research Center, Food Safety and Technologies Initiative, Glenside, PA

Shaunti Luce, The National Food Lab, Livermore, CA

Hondo Dammann and Dorn Clark Jr, Marshfield Food Safety, Marshfield, WI

Wendy McMahon and Deena Awad, Silliker, Inc., Crete, IL

Michelle Kelly and Megan Greenwell, Q Laboratories Inc., Cincinnati, OH

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FOOD BIOLOGICAL CONTAMINANTS

Evaluation of VIDAS® UP *Listeria* Assay (LPT) for the Detection of *Listeria* in a Variety of Foods and Environmental Surfaces: First Action 2013.10

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The VIDAS® UP *Listeria* (LPT) is an automated rapid screening enzyme phage-ligand based assay for the detection of *Listeria* species in human food products and environmental samples. The VIDAS LPT method was compared in a multi-laboratory collaborative study to AOAC Official Method 993.12 *Listeria monocytogenes* in Milk and Dairy Products reference method following current AOAC guidelines. A total of 14 laboratories participated, representing government and industry, throughout the United States. One matrix, queso fresco (soft Mexican cheese), was analyzed using two different test portion sizes, 25 and 125 g. Samples representing each test portion size were artificially contaminated with *Listeria* species at three levels, an uninoculated control level [0 colony-forming units (CFU)/test portion], a low-inoculum level (0.2–2 CFU/test portion), and a high-inoculum level (2–5 CFU/test portion). For this evaluation, 1800 unpaired replicate test portions were analyzed by either the VIDAS LPT or AOAC 993.12. Each inoculation level was analyzed using the Probability of Detection (POD) statistical model. For the low-level inoculated test portions, difference in collaborator POD (dLPOD) values of 0.01, (–0.10, 0.13), with 95% confidence intervals, were obtained for both 25 and 125 g test portions. The range of the confidence intervals for dLPOD values for both the 25 and 125 g test portions contains the point 0.0 indicating no statistically significant difference in the number of positive samples detected between the VIDAS LPT and the AOAC methods. In addition to Oxford agar, VIDAS LPT test portions were confirmed using Agar Listeria Ottavani and Agosti (ALOA), a proprietary chromogenic agar for the identification and differentiation of *L. monocytogenes* and *Listeria* species. No differences were observed between the two selective agars. The VIDAS LPT method, with the optional ALOA agar confirmation method, was adopted as Official First Action status for the detection of *Listeria* species in a variety of foods and environmental samples.

Submitted for publication November 1, 2013.
The method was approved by the Expert Review Panel for Microbiology Methods for Feed and Environmental Surfaces as First Action.
The Expert Review Panel for Microbiology Methods for Feed and Environmental Surfaces invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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Supplemental data is available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac and follow link to supplemental data.
DOI: 10.5740/jaoacint.13-372

The current classification of the genus *Listeria* includes six well-characterized species, with *L. monocytogenes* being the species of most concern in foodborne outbreaks (1). *Listeria* species are short, non-spore forming Gram-positive rods that are ubiquitous in the environment and can be found in soil, decaying vegetation, and most environments (2). While the number of people who become ill from listeriosis, the disease caused by *Listeria,* is relatively small, the high mortality rate from infection makes it one of the leading causes of death from foodborne illness (2). Of primary concern for illness from *Listeria* outbreaks are the elderly, pregnant women, infants, and people with compromised immune systems (3). Outbreaks from *Listeria* have been linked to such foods as ready-to-eat deli meats, hot dogs, pâtés, dairy products, soft cheeses, smoked seafood, raw sprouts, and most recently cantaloupes (4). The VIDAS UP *Listeria* (LPT) assay, an automated enzyme phage-ligand based assay for the screening of *Listeria* in food and environmental samples, provides the ability to detect *Listeria* after only 26 h of enrichment.

The VIDAS LPT assay uses a primary enrichment (prewarmed to 18–25°C) to detect *Listeria* species in 25 g test portions after 26–30 h of enrichment. For cantaloupe melons, whole melons are soaked in approximately 1 L LPT broth and incubated following conditions outlined for 25 g test portions. For larger

samples sizes, such as 125 g, following 24–30 h primary enrichment incubation, a transfer to a secondary enrichment in 10 mL LPT broth and an additional 22–26 h of incubation is required prior to detection. For smaller test portion sizes and cantaloupe melons, the new enrichment method eliminates the need for secondary enrichments and produces negative and presumptive positive results the following day.

Prior to the collaborative study, the VIDAS LPT method was validated by expert laboratories according to *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, Appendix J (5) in a precollaborative study. The objective of this study was to demonstrate that the VIDAS LPT method could detect *Listeria* spp. in a variety of foods and environmental surfaces as claimed by the manufacturer. For the VIDAS LPT evaluation, 19 matrixes were tested: deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), regular peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), and stainless steel, plastic, ceramic, and concrete environmental surfaces.

During the precollaborative method comparison evaluation, 525 unpaired samples were analyzed by the VIDAS LPT method. One false-positive result and 0 false-negative results were observed. Using the POD statistical model, no significant difference was observed between the reference method and the VIDAS LPT method for all matrixes analyzed except bagged mixed salad, beef hot dogs, and stainless steel environmental samples. For these three matrixes, the VIDAS LPT detected significantly more positive samples than the reference method, which resulted in the statistically significant difference. The inclusivity and exclusivity evaluation showed no unexpected results. The VIDAS LPT method detected all of the *Listeria* strains analyzed and none of the non-*Listeria* strains analyzed. The precollaborative data and report were reviewed by an expert review panel (ERP) prior to approval of the AOAC collaborative protocol. The precollaborative data are presented as supplemental data on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

This collaborative study compared the VIDAS LPT method to the AOAC **993.12** *Listeria monocytogenes* in Milk and Dairy Products (6) method for queso fresco at two test portion sizes, 25 and 125 g.

Collaborative Study

Study Design

For this collaborative study, one matrix, queso fresco, was analyzed using two test portion sizes: 25 and 125 g. The queso fresco was obtained from local retailers and screened for the absence of *Listeria* by AOAC **993.12** prior to analysis. The 25 and 125 g test portions of queso fresco were each inoculated with a different strain of *Listeria* at two inoculation levels: a high-inoculation level of approximately 2–5 colony-forming units (CFU)/test portion and a low-inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions were also included for each matrix at 0 CFU/test portion. The 25 g test portions were artificially contaminated with *L. innocua* ATCC 33090 and the 125 g test portions with *L. monocytogenes* ATCC 19115.

Twelve replicate portions from each of the three inoculation levels of product were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by VIDAS LPT and AOAC **993.12** due to different sample enrichments for each method.

A detailed collaborative study packet outlining all necessary information related to the study, including media preparation, method-specific test portion preparation, and documentation of results, was sent to each collaborating laboratory prior to the initiation of the study.

Preparation of Inocula and Test Portions

The *Listeria* cultures used in this evaluation were propagated in 10 mL brain heart infusion (BHI) broth from a frozen stock culture stored at –70°C at Q Laboratories, Inc. The broth was incubated for 18–24 h at 35 ±1°C. The inoculum was heat stressed in a 50 ± 1°C water bath for 10 min to obtain a percent injury of 50–80%, as determined by plating onto selective Oxford agar (OXA) and nonselective trypticase soy agar (TSA). The degree of injury was estimated as

$$(1-\frac{n_{select}}{n_{nonselect}})x100$$

where n_{select} = number of colonies on selective agar and $n_{nonselect}$ = number of colonies on nonselective agar. Appropriate dilutions of the heat-stressed cultures were prepared based on previously established growth curves for both low- and high-inoculation levels, resulting in fractional positive outcomes for at least one level. For both test portion sizes, a bulk lot of the queso fresco was inoculated with a liquid inoculum and mixed thoroughly by hand kneading to ensure an even distribution of microorganisms. The queso fresco was inoculated on the day of shipment so that all test portions would have been held for 96 h by the day testing was initiated. The shipment and hold times of the inoculated test material had been verified through 120 h as a quality control measure prior to study initiation. For the analysis of the 25 g test portions, the bulk lot of test material was divided into 30 g portions for shipment to the collaborators. For the analysis of the 125 g test portions, 25 g of inoculated test product was mixed with 100 g of uninoculated test product for shipment to the collaborators for the analysis by the VIDAS LPT method. Collaborators received 30 g portions for analysis by AOAC **993.12**. Validation criterion is satisfied when inoculated test portions produce fractional recovery of the spiked organism, defined as either the reference or candidate method yielding 25–75% positive results. To determine the level of *Listeria* spp. in the queso fresco, a 5-tube most probable number (MPN) was conducted on the day of initiation of analysis. From both the high- and low-inoculated batches of queso fresco, five 100 g test portions, the reference method test portions from the collaborating laboratories, and five 10 g test portions were analyzed following AOAC **993.12**. The MPN and 95% confidence intervals were calculated from the high, low, and uninoculated levels using the Least Cost Formulations (LCF; Norfolk, VA) MPN Calculator provided by AOAC (7).

Confirmation of the samples was conducted according to AOAC **993.12**.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3-digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transportation Association. Upon receipt, samples were held by the collaborating laboratory at refrigeration temperature (3–5°C) until the following Monday when analysis was initiated. All samples were packed with cold packs to target a temperature of <7°C during shipment. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as 'temperature control'. Participants were instructed to obtain the temperature of this portion upon receipt of the package, document results on the Sample Receipt Confirmation form provided, and fax to the study director.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method for each test portion size. For both test portion sizes, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls for each evaluation). For the analysis of the 25 g test portions by VIDAS LPT, a 25 g sample replicate was enriched with 225 mL prewarmed (18–25°C) LPT broth and homogenized for 2 min. Test portions were incubated for 26–30 h at 30 ± 1°C. For the 125 g test portions analyzed by VIDAS LPT, a 125 g sample replicate was enriched with 375 mL prewarmed (18–25°C) LPT broth and homogenized for 2 min. Test portions were incubated for 24–30 h at 30 ± 1°C. For 125 g test portions, a 1.0 mL aliquot of the primary enrichment was transferred into 10 mL LPT broth and incubated for an additional 22–26 h at 30 ± 1°C.

Following enrichment, samples were assayed by VIDAS LPT and confirmed following procedures outlined in the standard reference method by streaking an aliquot of the primary enrichment onto OXA and a proprietary chromogenic agar, ALOA. Presumptive positive samples were streaked for isolation on TSA yeast extract (TSAYE) and biochemically confirmed by morphology verification via Gram stain, hemolysis test, and by AOAC **2012.02** VITEK 2 GP Biochemical Identification method (VITEK 2 GP) or API *Listeria* (1) biochemical test kits. Laboratories utilizing API *Listeria* kits were also required to conduct a catalase test and an oxidase test.

Both test portion sizes analyzed by the VIDAS LPT methods were compared to samples (25 g) analyzed using the AOAC **993.12** reference method in conjunction with VITEK 2 GP or API *Listeria* for the confirmation of *Listeria* in an unpaired study design. Twenty-five gram test portions were enriched in prewarmed (45°C) selective enrichment broth, homogenized for 2 min, and incubated at 30 ± 2°C for 48 h. Samples were streaked onto OXA and presumptive positive samples were streaked for isolation onto TSAYE. Colonies from TSAYE were confirmed by morphology verification via Gram stain, hemolysis test, and by VITEK 2 GP or API *Listeria* kits. Laboratories utilizing API *Listeria* kits were also required to conduct a catalase test and an oxidase test.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and VIDAS LPT results. The data sheets were submitted to the study director for analysis at the end of each week. The results of each test portion for each sample were compiled by the study director and the qualitative VIDAS LPT results were compared to the reference method for statistical analysis. Data for each test portion size was analyzed using the POD statistical model (5, 8). For each inoculation level, the probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials. The POD was calculated for the candidate presumptive results, POD_{CP}, the candidate confirmatory results, POD_{CC}/POD_{C}, the reference method, POD_{R}, the difference in the candidate presumptive and confirmatory results, $dLPOD_{CP}$, and the difference in the candidate confirmed and reference methods, $dLPOD_{C}$. A confidence interval of a dLPOD not containing the point zero would indicate a statistically significant difference between VIDAS LPT and AOAC **993.12** at the 5% probability level (9).

AOAC Official Method 2013.10
Listeria species in a Variety of Foods and Environmental Surfaces
VIDAS® UP *Listeria* (LPT) Method
First Action 2013

[Applicable to detection of *Listeria* in deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), stainless steel, plastic, ceramic and concrete environmental surfaces.]

See Tables **2013.10A** and **B** for a summary of results of the collaborative study. *See* supplemental data, Tables 2A–D, for detailed results of the collaborative study on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Caution: *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these concerned groups avoid handling this organism. Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. Some reagents in the kit contain 1 g/L concentrations of sodium azide. Check local regulations prior to disposal. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards. This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is, therefore, recommended that these products be treated as potentially infectious and

Table 2013.10A. Summary of results for the detection of *Listeria* spp. in queso fresco (25 g)

Method[a]	VIDAS LPT w/OXA			VIDAS LPT w/ALOA		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. samples analyzed	1/156	80/156	156/156	1/156	80/156	156/156
Candidate presumptive POD (CP)	0.01	0.51	1.00	0.01	0.51	1.00
	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)	(0.01, 0.04)	(0.43, 0.59)	(0.98, 1.00)
s_r[b]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.07, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)	(0.00, 0.03)	(0.00, 0.13)	(0.00, 0.15)
s_R[d]	0.08	0.51	0.00	0.08	0.51	0.00
	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)	(0.07, 0.13)	(0.46, 0.52)	(0.00, 0.21)
P value[e]	0.4395	0.9210	1.0000	0.4395	0.9210	1.0000
Candidate confirmed positive/total No. samples analyzed	0/156	78/156	156/156	0/156	78/156	156/156
Candidate confirmed POD (CC)	0.00	0.50	1.00	0.00	0.50	1.00
	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)	(0.00, 0.02)	(0.42, 0.58)	(0.98, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)
P value	1.0000	0.9161	1.0000	1.0000	0.9161	1.0000
Positive reference samples/total No. samples analyzed	0/156	76/156	156/156	0/156	76/156	156/156
Reference POD	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)	(0.00, 0.02)	(0.41, 0.57)	(0.98, 1.00)
s_r	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.10)	(0.00, 0.15)
s_R	0.00	0.52	0.00	0.00	0.52	0.00
	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)
P value	1.0000	0.9937	1.0000	1.0000	0.9937	1.0000
dLPOD (candidate vs reference)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)
dLPOD (candidate presumptive vs candidate confirmed)	0.01	0.01	0.00	0.01	0.01	0.00
	(–0.02, 0.04)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.04)	(–0.10, 0.13)	(–0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

handled observing the usual safety precautions (do not ingest or inhale).

A. Principle

VIDAS® UP *Listeria* (LPT) method is for use on the automated VIDAS instrument for the detection of *Listeria* antigens using the enzyme-linked fluorescent assay (ELFA) method. The assay also incorporates phage proteins allowing an increase in sensitivity and specificity compared to traditional immunoassay. The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device. The interior of the SPR is coated with proteins specific for *Listeria* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent

Table 2013.10B. Summary of results for the detection of *Listeria* spp. in queso fresco (125 g)

Method[a]	VIDAS LPT w/OXA			VIDAS LPT w/ALOA		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value[e]	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate confirmed POD (CC)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Positive reference samples/total No. of samples analyzed	0/144	69/144	144/144	0/144	69/144	144/144
Reference POD	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9672	1.0000	1.0000	0.9672	1.0000
dLPOD (C vs R)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)
dLPOD (CP vs CC)	0.00	0.00	0.00	0.00	0.00	0.00
	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *Listeria* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Listeria* receptors, which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the

Table 2013.10C. Reagents included in 10-well reagent strip

Wells	Reagents (LPT)
1	Sample well: 0.5 mL of enrichment broth, standard or control
2	Prewash solution (400 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
3–5, 7–9	Wash buffer (600 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
6	Conjugate (400 µL): alkaline phosphatase-labeled proteins specific for *Listeria* receptors + preservative
10	Reading cuvette with substrate (300 µL): 4-Methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA) (0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent: *See* VIDAS LPT package insert for more information.

substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument, which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS UP *Listeria* (LPT) assay kit from bioMérieux (595 Anglum Rd, Hazelwood, MO 63042-2330, USA).

(**a**) *VIDAS or miniVIDAS automated immunoassay system*.

(**b**) *LPT reagent strips.*—Sixty polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents shown in Table **2013.10C**.

(**c**) *SPR.*—Sixty SPRs coated with proteins specific for *Listeria* receptors.

(**d**) *Standard.*—One vial (1 × 6 mL). Ready-to-use. Contains purified and inactivated *Listeria* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—1 × 6 mL. Contains purified and inactivated *Listeria monocytogenes* antigen + preservative + protein stabilizer.

(**f**) *Negative control solution.*—1 × 6 mL. Contains Tris-buffered saline (TBS; 150 mmol/l) – Tween pH 7.6 + preservative.

(**g**) *Master Lot Entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test: To read the MLE data, please refer to the Operator's Manual.

(**h**) *Package insert*.

(**i**) *Disposable pipet.*—To dispense appropriate volumes.

(**j**) *VIDAS Heat and Go.*—Available from bioMérieux, Inc.

(**k**) *Water bath.*—95–100°C, or equivalent.

(**l**) *Bag with filter.*

(**m**) Smasher™ Blender/Homogenizer available from bioMérieux, Inc., or equivalent.

(**n**) *LPT broth.*—bioMérieux, Inc.

(**o**) *Incubators.*—Capable of maintaining 30 ± 1°C and 35 ± 1°C.

(**p**) *Diagnostic reagents.*—Necessary for culture confirmation of assays.

(**q**) *ALOA chromogenic agar.*—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**r**) *Tryptic Soy Agar with yeast additive*.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS LPT kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Standard, controls, and heated test portions are mixed well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* safety precautions in the VIDAS LPT package insert (Warnings and Precautions and Waste Disposal).

(**i**) *See* Centers for Disease Control recommendations in handling pathogens. http:/www.cdc.gov/biosafety/publications/bmb15/index.htm/

D. Preparation of Test Suspension

(**a**) *Pre-enrichment.*—Pre-enrich test portion using filter Stomacher type bags to initiate growth of *Listeria.* For 25 g test portions, add 225 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min. For cantaloupe melons, soak entire melon in approximately 1 L prewarmed (18–25°C) LPT broth. For 125 g test portions, add 375 mL prewarmed (18–25°C) LPT broth to each test portion and homogenize thoroughly for 2 min.

(**b**) *Test portions.—(1) 25 g test portions/cantaloupe melons rinses.*—After homogenization, incubate for 26–30 h at 30 ± 1°C.

(2) 125 g test portions.—After homogenization, incubate for 24–30 h at 30 ± 1°C.

From the primary enrichment broth, transfer a 1 mL aliquot into 10 mL prewarmed (18–25°C) LPT broth and incubate for 22–26 h at 30 ± 1°C.

(**c**) After incubation, homogenize samples manually. Follow appropriate instructions based on heating method.

(*1*) *Boiling.*—Transfer 2–3 mL of the enrichment broth into a tube. Seal the tube. Heat in a water bath for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.5 mL into the sample well of the VIDAS LPT reagent strip. Perform the VIDAS test.

(2) Heat and Go.—Transfer 0.5 mL of the enrichment broth into the sample well of the VIDAS LPT reagent strip. Heat for 5 ± 1 min (*See* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

Table 2013.10D. Interpretation of test

Test value threshold	Interpretation
<0.05	Negative
≥0.05	Positive

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and let them to come to room temperature for at least 30 min.

(**c**) Use one VIDAS LPT reagent strip and one VIDAS LPT SPR for each sample, control, or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "LPT," and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1." If the negative control is to be tested, identify it by "C2."

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the LPT reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, the test sample identification, the date and time, the lot number and expiration date of the reagent kit being used, and each sample's RFV, test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A "negative" result has a test value less than the threshold (0.05) and indicates that the sample does not contain *Listeria* spp. or contains *Listeria* spp. at a concentration below the detection limit. A "positive" result has a test value equal to or greater than the threshold (≥0.05) and indicates that the sample may be contaminated with *Listeria* spp. If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2013.10D**).

G. Confirmation

All positive VIDAS LPT results must be culturally confirmed. Confirmation should be performed using the nonheated enrichment broth stored between 2–8°C, and should be initiated within 72 h following the end of incubation (AFNOR Certificate No. BIO 12/33-05/12). Presumptive positive results may be confirmed by isolating on selective agar plates such as ALOA or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in appropriate reference method. As an alternative to the conventional confirmation for *Listeria*, AOAC **2012.02** VITEK 2 GP Biochemical Identification or API *Listeria* biochemical kits may be used for presumptive generic identification of foodborne *Listeria*.

Results of Collaborative Study

In this collaborative study, the VIDAS UP *Listeria* (LPT) method was compared to AOAC **993.12** for one food product, queso fresco, at two test portion sizes: 25 and 125 g. A total of 14 laboratories throughout the United States participated in this study, with 14 laboratories submitting data for the 25 g test portions and 13 laboratories submitting data for the 125 g test portions as presented in Table 1. Each laboratory analyzed 36 test portions for each method—12 inoculated with a high level of *Listeria*, 12 inoculated with a low level of *Listeria*, and 12 uninoculated controls. A background screen of the matrix indicated an absence of indigenous *Listeria* species. As per criteria outlined in Appendix J of the AOAC guidelines, fractional positive results were obtained for both the 25 and 125 g test portions sizes. Cultures used to inoculate the matrix were heat stressed, and the results of the inoculum heat stress are presented in Table 2. For each test portion size, the actual level of *Listeria* was determined by MPN determination on the day of initiation of analysis. The individual laboratory and sample results are presented in Tables 3 and 4. Tables **2013.10A** and **2013.10B** summarize the collaborative study results for all foods tested, including POD statistical analysis (8). Detailed results for each

Table 1. Participation of each collaborating laboratory[a]

	Queso fresco	
Lab	25 g test portions	125 g test portions
1	Y	Y
2	Y	Y[b]
3	Y	Y
4	Y	Y
5	Y	Y
6	Y	Y
7	Y	Y
8	Y	Y
9	Y	Y
10	Y	Y
11	Y[c]	Y[c]
12	Y	Y
13	Y	Y
14	Y	Y

[a] Y = Collaborator analyzed the food type.

[b] Results were not submitted to the coordinating laboratory.

[c] Results were not used in statistical analysis due to laboratory error.

Table 2. Heat-stress injury

Matrix	Test organism	CFU/OXA (selective agar)	CFU/TSA (nonselective agar)	Degree injury, %
Queso fresco	*L. innocua*	5.3×10^8	1.3×10^9	59
LPT – 25 g	ATCC[a] 33091			
Queso fresco	*L. monocytogenes*	2.9×10^8	9.0×10^8	68
LPT – 125 g	ATCC 19115			

[a] ATCC = American Type Culture Collection.

laboratory are presented in Tables 2A–D, and Figures 1A–D and 2A–D as supplemental data on *J. AOAC Int.* website.

Queso Fresco (25 g Test Portions)

Queso fresco test portions, inoculated at a low and high levels, were analyzed for the detection of *Listeria* spp. (Table 3). Uninoculated controls were included in each sample set. Fourteen laboratories participated in the analysis of this matrix, and the results of 13 laboratories were included in the statistical analysis. Laboratory 11 reported data for eight reference method test portions (including seven uninoculated control test portions) that produced doubtful profiles of *L. grayi*. Colonies on these plates were also reported as beta-hemolytic, a characteristic not associated with *L. grayi.* The selective agar plates for these test portions were sent to the coordinating laboratory for further examination. Colonies present on the plates did not possess characteristics typical of *Listeria* spp. Colonies were identified as Gram-positive rods containing spores with morphology typical of *Bacillus* species. Based on the preliminary biochemical tests conducted, the test portions should not have been carried through for final biochemical identification on API *Listeria* strips, which resulted in the misidentification of the test portion as *Listeria* spp. The results for this laboratory were excluded from statistical analysis. The MPNs obtained for this matrix, with 95% confidence intervals, were 0.63 CFU/test portion (0.49, 0.79) for the low-inoculum level and 5.48 CFU/test portion (3.60, 8.36) for the high-inoculum level. For VIDAS LPT test portions, no differences were observed between confirmation of samples using the proprietary chromogenic ALOA and the reference method agar.

For the high-inoculum level, 156 out of 156 test portions were reported as positive by the VIDAS LPT method with all test portions confirming positive. For the low-inoculum level, 80 out of 156 test portions were reported as positive by the VIDAS LPT method with 78 test portions confirming positive, indicating two false-positive results. For the uninoculated controls, 1 out of 156 samples produced a presumptive positive result by the VIDAS LPT method with no samples confirming positive. All three false-positive samples were obtained from the same laboratory. For test portions analyzed by AOAC **993.12**, 156 out of 156 high-inoculum test portions and 76 out of 156 low-inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 156 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.01 (–0.10, 0.13) was obtained between AOAC **993.12** and VIDAS LPT. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.01 (–0.10, 0.13) was obtained between presumptive and confirmed VIDAS LPT results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.00 (–0.02, 0.02) was obtained between AOAC **993.12** and VIDAS LPT. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.00 (–0.02, 0.02) was obtained between presumptive and confirmed VIDAS LPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Results of the POD statistical analysis are presented in Table **2013.10A**, Tables 2A–B, and Figures 1A–D, as supplemental data on the *J. AOAC Int.* website.

Queso Fresco (125 g Test Portions)

Queso fresco test portions were inoculated at a low and high level and analyzed for the detection of *Listeria* spp. (Table 4). Uninoculated controls were included in each sample set. Fourteen laboratories participated in the analysis of this matrix, and the results of 12 laboratories were included in the statistical analysis. Laboratory 2 did not report any data for this matrix. Laboratory 11 reported 10 reference method test portions (including five uninoculated control test portions) that produced non-*L. monocytogenes* profiles, with five of the test portions producing doubtful profiles of *L. grayi*. Colonies on these plates contained one or more of the following biochemical reactions not typically associated with *L. monocytogenes*: Gram-negative, non-beta-hemolytic, and catalase negative. Based on the preliminary biochemical tests conducted, the test portions should not have been carried through for final biochemical identification on API *Listeria* strips which resulted in the misidentification of the test portion as *Listeria* spp. The selective agar plates for these test portions were sent to the coordinating laboratory for further examination. The coordinating laboratory confirmed the supplementary results (Gram stain, hemolysis, and catalase reaction) reported by the participating laboratory and were not able to identify any *Listeria* species. The results from this laboratory were excluded from statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.59 CFU/test portion (0.46, 0.74) for the low level and 5.41 CFU/test portion (3.53, 8.30) for the high level. For VIDAS LPT test portions, no differences were observed between confirmation of samples using the proprietary chromogenic ALOA and the reference method agar.

For the high level, 144 out of 144 test portions were reported as positive by the VIDAS LPT method with all test portions confirming positive. For the low level, 70 out of 144 test portions were reported as positive by the VIDAS LPT method with all 70 test portions confirming positive. For the uninoculated controls, 0 out of 144 samples produced a presumptive positive result by the VIDAS LPT method and no samples confirming positive. For test portions analyzed by AOAC **993.12**, 144 out of 144 high inoculum and 69 out of 144 low inoculum test

Table 3. Individual collaborator results for queso fresco[a] (25 g test portions)

	VIDAS LPT[b]																																	
	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	–	–	+	+	–[d]	–[d]	–	–	–	–	–	–	–	–	–[d]	–	–	–
	AOAC **993.12**																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	–	+[e]	+	+	+	+	–	–	+	–	+	+	+[e]	+[e]	+[e]	–	–	–	+[e]	+[e]	–	–	+[e]	+[c]
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Listeria* spp. were detected in samples; – = *Listeria* spp. were not detected in sample.

[b] Confirmed results from OXA and ALOA were identical for each test portion.

[c] Results were not used in statistical analysis.

[d] Sample was presumptive positive on VIDAS LPT but confirmed negative indicating a false-positive result.

[e] Result reported as *L. grayi* (doubtful API Profile).

portions confirmed positive. For the uninoculated controls, 0 out of 144 test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.01 (–0.10, 0.13) were obtained between AOAC **993.12** and VIDAS LPT. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.12, 0.12) were obtained between presumptive and confirmed VIDAS LPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of 0.00 (–0.03, 0.03) were obtained between AOAC **993.12** and VIDAS LPT. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.03, 0.03) were obtained between presumptive and confirmed VIDAS LPT results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.10B**, as supplemental data on the *J. AOAC Int.* website, Tables 2C–D, and Figures *2*A–D.

ALOA Chromogenic Agar

Confirmatory results obtained from the ALOA chromogenic

Table 4. Individual collaborator results for queso fresco[a] (125 g test portions)

	VIDAS LPT[b]																																	
	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
	AOAC **993.12**																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	+	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	+[d]	+	+[e]	+	+	+[f]	+[f]	–	–	+[f]	+	+	–	–	+[f]	+[e]	–	–	–	–	–	+[g]	+[g]	+[f]
12	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Listeria* spp. were detected in samples; – = *Listeria* spp. were not detected in sample; NA laboratory did not participate in this matrix or results were not received.

[b] Confirmed results from OXA and ALOA were identical for each test portion.

[c] Results not used in statistical analysis.

[d] Result reported as *L. welshimeri.*

[e] Result reported as *L. innocua.*

[f] Result reported as *L. grayi.*

[g] Result reported as *L. ivanovii.*

agar were identical to the results obtained from the OXA agar specified by AOAC **993.12**. Out of a total of 451 positives detected by the VIDAS LPT, 448 were confirmed positive using OXA or ALOA selective agars.

Discussion

No negative feedback was reported to the study directors from the collaborating laboratories in regards to the performance of the VIDAS LPT assay or the ALOA chromogenic agar. Many laboratories indicated difficulty in identifying and isolating colonies from samples when using OXA plates, but not from test portions analyzed by the VIDAS LPT method. These results may be due to the higher selectivity of the ALOA agar to isolate and differentiate typical *Listeria* colonies from competing microflora, such as *Bacillus* colonies. The high selectivity of the proprietary LPT broth, the high background flora, and the low selectivity of the OXA agar most likely contributed to this observation, as well.

For the analysis of 25 g test portions by the VIDAS LPT method, three false positives were obtained. The test results produced by three false-positive test portions (average test value of 0.34) were much lower than the test values observed with true positives (average value >2.00). By the time the coordinating laboratory received the results, the primary enrichments for these samples had been discarded so no subsequent analysis on the VIDAS LPT was possible. However, the agar plates for these test portions were shipped to the coordinating laboratory for further analysis. Up to 20 different colonies were picked for morphological and biochemical analysis using VITEK 2 GP and no *Listeria* colonies were identified. Additionally, the entire lawn of growth from each agar plate was swabbed and enriched in separate LPT broth tubes and incubated for 26–30 h at 30 ± 1°C. An aliquot from each tube was analyzed by the VIDAS LPT assay and negative results for *Listeria* spp. were obtained. Results of this investigation lead the study directors to believe that the false positives were the result of contamination during the analysis of the samples.

For the analysis of both the 25 and 125 g test portions, Laboratory 11 detected the presence of multiple species of *Listeria*. An investigation into the results indicated that colonies picked for confirmation did not meet the characteristics of *Listeria* spp. (i.e., colonies produced Gram-negative stain reactions, non-motile, negative catalase, or produced hemolysis reactions not typically observed with *Listeria* spp.). The results of these tests should have precluded analysis using the API strips, which lead to an inaccurate identification. Due to the fact that final results reported were inconsistent with biochemical results, data produced by Laboratory 11 were removed from the statistical analysis of both the 25 and 125 g test portions.

Typical growth of *Listeria* spp. colonies from ALOA was easy to identify and the ALOA plates produced less background ground from the matrix than the OXA plates for both test portions sizes analyzed. Positive comments were received from collaborators about the ease of use associated with the ALOA plates.

Using the POD statistical model, no significant difference in the number of positive results obtained between the two methods being compared was observed at both the low- and high-inoculum levels for both the 25 and 125 g test portions. No significant difference was observed between presumptive and confirmed results for the candidate method.

Conclusions

The VIDAS UP *Listeria* (LPT) method with the optional ALOA agar confirmation method was adopted as Official First Action status for the detection of *Listeria* in a variety of foods and environmental surfaces including deli ham (25 and 125 g), pepperoni (25 g), beef hot dogs (25 g), chicken nuggets (25 g), chicken liver pâté (25 g), ground beef (125 g), deli turkey (125 g), cooked shrimp (25 g), smoked salmon (25 g), whole cantaloupe melon, bagged mixed salad (25 g), peanut butter (25 g), black pepper (25 g), vanilla ice cream (25 g), queso fresco (25 and 125 g), stainless steel, plastic, ceramic, and concrete environmental surfaces.

Acknowledgments

We would like to extend a sincere thank you to the following collaborators for their dedicated participation in this study:

John Mills and Pat Rule, bioMérieux Industry (Hazelwood, MO)

Ben Howard, Neil Rogman, and Jacob Cannon, Certified Laboratories (Bollingbrook, IL)

Barbara Paul, Marianna Sala-Rhatigan, and Susan Joseph, U.S. Food and Drug Administration, Northeast Regional Laboratory (Jamaica, NY)

Nikki Palen, Amber Stegmann, and Bryan Perry, EMS Laboratories (St. Louis, MO)

Rachel Hiles and Tenesha Stubblefield, Silliker Laboratories (Omaha, NE)

Nigel Nagassar, Sylvanus Owusu, and Jacqueline Zimmerman, Silliker Laboratories (Minnetoka, MN)

Jerry Lynn Pickett, Aaron Bollenbacher, Keith Wiggins, and Lori Cesanas, Tyson WBA Analytical (Springdale, AR)

Bharath Brahmanda, Food Safety Net Services (San Antonio, TX)

Andrew Capps, Grisel Rosario, Dawn Davis, Lindsey Parker, Christine Said, and Jianfeng Li, North Carolina Department of Agriculture and CS: Food and Drug Protection Division (Raleigh, NC)

Keith Klemms, Bill May, Becky Hand, and Rose Burkhart, Sherry Laboratories (Warsaw, IN)

Hesham Elgaali, Indiana State Department of Health, Food and Dairy Microbiology Division (Indianapolis, IN)

Jennifer Jolly, Covance Laboratories (Battle Creek, MI)

Sandy Moore, Dustin Ebbing, Maggie Michels, Amanda Kehres, and Joe Hirsch, John Morrell (Springdale, OH)

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FOOD BIOLOGICAL CONTAMINANTS

Evaluation of VIDAS® *Listeria monocytogenes* Xpress (LMX) for the Detection of *Listeria monocytogenes* in a Variety of Foods: First Action 2013.11

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The VIDAS® *Listeria monocytogenes* Xpress (LMX) is an automated rapid screening enzyme immunoassay for the detection of *Listeria monocytogenes* in food products. The VIDAS LMX method was compared in a multi-laboratory collaborative study to AOAC Official Method 993.12 *Listeria monocytogenes* in Milk and Dairy Products reference method following current AOAC guidelines. A total of 14 laboratories participated, representing government and industry, throughout the United States. One matrix, queso fresco (soft Mexican cheese), was analyzed using two different test portion sizes, 25 and 125 g. Samples representing each portion size were artificially contaminated with *L. monocytogenes* at three levels: an uninoculated control level [0 colony forming units (CFU)/test portion], a low inoculum level (0.2–2 CFU/test portion), and a high inoculum level (2–5 CFU/test portion). For this evaluation, 1800 unpaired replicate test portions were analyzed by either the VIDAS LMX or AOAC 993.12. Each level was analyzed using the Probability of Detection (POD) statistical model. For the low-level inoculated test portions, difference in collaborator POD (dLPOD) values of 0.04, (–0.08, 0.15) and 0.01, (–0.10, 0.13), with 95% confidence intervals, were obtained, respectively, for 25 and 125 g test portions. The range of the confidence intervals for dLPOD values for both the 25 and 125 g test portions contain the point 0.0 indicating no statistically significant difference in the number of positive samples detected between the VIDAS LMX and the AOAC method. In addition to Oxford Agar (OXA), VIDAS LMX test portions were confirmed using Agar Listeria Ottavani and Agosti (ALOA), a proprietary chromogenic agar for the identification and differentiation of *L. monocytogenes* and *Listeria* species. No differences were observed between the two selective agars. The VIDAS LMX method, with the optional ALOA agar confirmation method, was adopted as Official First Action status for the detection of *L. monocytogenes* in a variety of foods.

Listeria monocytogenes is found widespread throughout the environment, having been isolated from soil, vegetation, marine sediments, and water as well as many different types of food products (1). While *L. monocytogenes* has long been known to cause illness in animals, it has only more recently been identified as the cause of listeriosis in humans (1). Listeriosis, while rare, can be of great concern for the elderly, pregnant women, infants, and the immunocompromised, as the disease can lead to septicemia, meningitis, encephalitis, or death (2, 3). Outbreaks from *L. monocytogenes* have been linked to such foods as ready-to-eat deli meats, hot dogs, pâtés, dairy products, soft cheese, smoked seafood, raw sprouts, and most recently cantaloupes (4). The VIDAS *Listeria monocytogenes* Xpress (LMX) assay, an automated enzyme-based assay for the screening of *L. monocytogenes* in food, provides the ability to rapidly detect the target analyte in only 1 to 2 days, depending on sample size.

The VIDAS LMX assay utilizes two proprietary enrichments to detect *L. monocytogenes* in food products, LMX broth with supplement for 25 g test portions and VIDAS UP *Listeria* (LPT) broth for 125 g test portions. The smaller test portions require 26–30 h of incubation, while larger test portions require a 24–30 h primary enrichment incubation followed by a secondary enrichment in 10 mL LPT broth for an additional 22–26 h of incubation. For smaller test portion sizes, the new enrichment

Submitted for publication October 31, 2013.
The method was approved by the Expert Review Panel for Microbiology Methods for Feed and Environmental Surfaces as First Action.

The Expert Review Panel for Microbiology Methods for Feed and Environmental Surfaces invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.

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Supplemental data is available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac and follow link to supplemental data.
DOI: 10.5740/jaoacint.13-368

method produces negative and presumptive positive results the next day after enrichment.

Prior to the collaborative study, the VIDAS LMX method was validated according to *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, Appendix J (5) in a harmonized AOAC *Performance Tested Method*^{SM} (PTM) study. The objective of this study was to demonstrate that the VIDAS LMX method could detect *L. monocytogenes* in a variety of foods as claimed by the manufacturer. For the VIDAS LMX evaluation, 11 matrixes were originally evaluated: processed cheese (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), smoked white fish (25 g), frozen spinach (25 g), peanut butter (25 g), and five "ready-to-eat" (RTE) 25 g meats (hot dogs, deli turkey, deli ham, fermented sausage, and pâtés). A matrix extension was conducted to evaluate four additional matrixes: deli ham (125 g), deli turkey (125 g), queso fresco (125 g), and ground beef (125 g).

All other PTM evaluation requirements (inclusivity, exclusivity, ruggedness, stability, and lot-to-lot variability) were satisfied. The method was awarded PTM certification No. 091103 on September 14, 2011 (6). The matrix extension was granted approval on January 15, 2013. This collaborative study compared the VIDAS LMX method to AOAC **993.12** *Listeria monocytogenes* in Milk and Dairy Products (7) method for queso fresco at two test portion sizes, 25 and 125 g.

Collaborative Study

Study Design

For this collaborative study, one matrix, queso fresco (soft Mexican cheese), was analyzed using two test portion sizes: 25 and 125 g. The queso fresco was obtained from local retailers and screened for the absence of *Listeria* by AOAC **993.12** prior to analysis. The 25 and 125 g test portions of queso fresco were inoculated with the same strain of *L. monocytogenes,* ATCC 19115, at two inoculation levels: a high inoculation level of approximately 2–5 colony-forming units (CFU)/test portion and a low inoculation level of approximately 0.2–2 CFU/test portion. A set of uninoculated control test portions were also included for each matrix at 0 CFU/test portion. Twelve replicate samples from each of the three inoculation levels of product were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by VIDAS LMX and AOAC **993.12** due to different sample enrichments for each method.

A detailed collaborative study packet outlining all necessary information related to the study, including media preparation, method-specific test portion preparation, and documentation of results, was sent to each collaborating laboratory prior to the initiation of the study.

Preparation of Inocula and Test Portions

The *L. monocytogenes* culture used in this evaluation was propagated in 10 mL brain heart infusion (BHI) broth from a frozen stock culture stored at –70°C at Q Laboratories, Inc. (Cincinnati, OH). The broth was incubated for 18–24 h at 35 ± 1°C. The inoculum was heat stressed in a 50°C water bath for 10 min to obtain a percent injury of 50–80%, as determined by plating onto selective Oxford agar (OXA) and nonselective Tryptic Soy agar (TSA). The degree of injury was estimated as

$$(1-\frac{n_{select}}{n_{nonselect}})x100$$

where n_{select} = number of colonies on selective agar and $n_{nonselect}$ = number of colonies on nonselective agar. Appropriate dilutions of the heat-stressed cultures were prepared based on previously established growth curves for both low and high inoculation levels, resulting in fractional positive outcomes for at least one level. For both test portion sizes, a bulk lot of the queso fresco was inoculated with a liquid inoculum and mixed thoroughly by hand kneading to ensure an even distribution of microorganisms. The queso fresco was inoculated on the day of shipment so that all test portions would have been held for 96 h by the day testing was initiated. The shipment and hold times of the inoculated test material had been verified through 120 h as a quality control measure prior to study initiation. For the analysis of the 25 g test portions by the VIDAS LMX and the AOAC **993.12** methods, the bulk lot of test material was divided into separate 30 g portions for shipment to the collaborators. For the analysis of the 125 g test portions by the VIDAS LMX method, 25 g of inoculated test product was mixed with 100 g of uninoculated test product for shipment to the collaborators. Validation criteria are satisfied when inoculated test portions produce fractional recovery of the spiked organism, defined as either the reference or candidate method yielding 25–75% positive results. To determine the level of *L. monocytogenes* in the queso fresco, a 5-tube most probable number (MPN) was conducted on the day of initiation of analysis. From both the high and low inoculated batches of queso fresco, five 100 g test portions, the reference method test portions from the collaborating laboratories, and five 10 g test portions were analyzed following AOAC **993.12**. The MPN and 95% confidence intervals were calculated from the high, medium, and low levels using the Least Cost Formulations (Norfolk, VA) MPN Calculator provided by AOAC (8). Confirmation of the samples was conducted according to AOAC **993.12**.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3-digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by International Air Transportation Association regulations. Upon receipt, samples were held by the collaborating laboratory at refrigeration temperature (3–5°C) until the following Monday when analysis was initiated. All samples were packed with cold packs to target a temperature of <7°C during shipment. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as 'temperature control'. Participants were instructed to obtain the temperature of this portion upon receipt of the package, document results on the Sample Receipt Confirmation form provided, and fax to the study director.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method for each test portion size. For both test portion sizes, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls per method). For the analysis of the 25 g test portions by the

VIDAS LMX method, each sample was enriched with 225 mL prewarmed (18–25°C) LMX broth containing LMX supplement (500 µL supplement/225 mL LMX broth) and homogenized for 2 min. Test portions were incubated for 26–30 h at 37 ± 1°C. For the 125 g test portions analyzed by the VIDAS LMX method, each sample was enriched with 375 mL prewarmed (18–25°C) LPT broth and homogenized for 2 min. Test portions were incubated for 24–30 h at 30 ± 1°C. For 125 g test portions only, a 1.0 mL aliquot of the primary enrichment was transferred into 10 mL LPT broth and incubated for 22–26 h at 30 ± 1°C.

Following enrichment, samples were assayed by VIDAS LMX and confirmed following procedures outlined in the reference method by streaking an aliquot of the primary enrichment onto OXA and a proprietary chromogenic agar, ALOA. Presumptive positive samples were streaked for isolation on TSA yeast extract (TSAYE) and biochemically confirmed by morphology verification via Gram stain, hemolysis test, and by VITEK 2 GP Biochemical Identification method (AOAC **2012.02**) or API *Listeria* biochemical test kits (9). Laboratories utilizing API *Listeria* kits were also required to conduct a catalase test and an oxidase test.

Both test portion sizes analyzed by VIDAS LMX were compared to 25 g portions analyzed using AOAC **993.12** in conjunction with VITEK 2 GP Biochemical Identification (AOAC **2012.02**) or API *Listeria* for the confirmation of *Listeria* in an unpaired study design. Twenty-five gram test portions were enriched in prewarmed (45°C) selective enrichment broth, homogenized for 2 min and incubated at 30 ± 2°C for 48 h. Samples were streaked onto OXA and presumptive positive samples were streaked for isolation onto TSAYE. Colonies from TSAYE were confirmed by morphology verification via Gram stain, hemolysis test, and by VITEK 2 GP Biochemical Identification method or API *Listeria* biochemical test kits. Laboratories utilizing API *Listeria* kits were also required to conduct a catalase test and an oxidase test.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and VIDAS LMX results. The data sheets were submitted to the study director at the end of each week of testing for analysis. The results of each test portion for each sample were compiled by the study director, and the qualitative VIDAS LMX results were compared to the reference method for statistical analysis. Data for each test portion size was analyzed using the POD statistical model. For each inoculation level, the probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials. The POD was calculated for the candidate presumptive results, POD_{CP}, the candidate confirmatory results, POD_{CC}/POD_{C}, the reference method, POD_{R}, the difference in the candidate presumptive and confirmatory results, $dLPOD_{CP}$, and the difference in the candidate confirmed and reference methods, $dLPOD_{C}$. A confidence interval of a dLPOD not containing the point zero would indicate a statistically significant difference between VIDAS LMX and AOAC **993.12** at the 5% probability level (10, 11).

AOAC Official Method 2013.11
Listeria monocytogenes in a Variety of Foods
VIDAS® *Listeria monocytogenes* Xpress (LMX) Method
First Action 2013

[Applicable to detection of *Listeria monocytogenes* in deli ham (25 and 125 g), fermented sausage (25 g), liver pâté (25 g), processed cheese (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), smoked white fish (25 g), frozen spinach (25 g), peanut butter (25 g), deli turkey (25 and 125 g), queso fresco (125 g), and ground beef (125 g).]

See Tables **2013.11A** and **B** for a summary of results of the collaborative study. *See* supplemental data, Tables 2A–D, for detailed results of the collaborative study on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Caution: *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these concerned groups avoid handling this organism. Dispose of all reagents and other contaminated materials by acceptable procedures for potentially biohazardous materials. Some reagents in the kit contain 1 g/L concentrations of sodium azide. Check local regulations prior to disposal. Disposal of these reagents into sinks with copper or lead plumbing should be followed immediately with large quantities of water to prevent potential hazards. This kit contains products of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not totally guarantee the absence of transmissible pathogenic agents. It is therefore recommended that these products be treated as potentially infectious and handled observing the usual safety precautions (do not ingest or inhale).

A. Principle

The VIDAS® *Listeria monocytogenes* Xpress (LMX) method is for use on the automated VIDAS instrument for the detection of *L. monocytogenes* antigens using the enzyme-linked fluorescent assay (ELFA) method. The Solid Phase Receptacle (SPR®) serves as the solid phase as well as the pipetting device. The interior of the SPR is coated with proteins specific for *L. monocytogenes* receptors. Reagents for the assay are ready-to-use and predispensed in the sealed reagent strips. All of the assay steps are performed automatically by the instrument. The reaction medium is cycled in and out of the SPR several times. An aliquot of enrichment broth is dispensed into the reagent strip. The *L. monocytogenes* receptors present will bind to the interior of the SPR. Unbound components are eliminated during the washing steps. The proteins conjugated to the alkaline phosphatase are cycled in and out of the SPR and will bind to any *Listeria monocytogenes* receptors which are themselves bound to the SPR wall. A final wash step removes unbound conjugate. During the final detection step, the substrate (4-methyl-umbelliferyl phosphate) is cycled in and out of the SPR. The conjugate enzyme catalyzes the hydrolysis of the substrate into a fluorescent product (4-methyl-umbelliferone), the fluorescence of which is measured at 450 nm. At the end of the assay, results are automatically analyzed by the instrument which calculates a test value for each sample. This value is then compared to internal references (thresholds) and each result is interpreted as positive or negative.

B. Apparatus and Reagents

Items (**a**)–(**h**) are available as the VIDAS *Listeria*

Table 2013.11A. Summary of results for the detection of *Listeria monocytogenes* in queso fresco (25 g)

Method[a]	VIDAS LMX w/OXA			VIDAS LMX w/ALOA		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. samples analyzed	0/156	77/156	156/156	0/156	77/156	156/156
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.02)	(0.41, 0.58)	(0.98, 1.00)	(0.00, 0.02)	(0.41, 0.58)	(0.98, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.12)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.12)	(0.00, 0.15)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.47, 0.52)	(0.00, 0.21)
P value[e]	1.0000	0.9772	1.0000	1.0000	0.9772	1.0000
Candidate confirmed positive/total No. samples analyzed	0/156	75/156	156/156	0/156	75/156	156/156
Candidate confirmed POD (CC)	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.02)	(0.40, 0.56)	(0.98, 1.00)	(0.00, 0.02)	(0.40, 0.56)	(0.98, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.00, 0.15)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)	(0.00, 0.15)	(0.00, 0.14)	(0.00, 0.15)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)	(0.00, 0.21)	(0.46, 0.52)	(0.00, 0.21)
P value	1.0000	0.8718	1.0000	1.0000	0.8718	1.0000
Positive reference samples/total No. samples analyzed	0/156	69/156	153/156	0/156	69/156	153/156
Reference POD	0.00	0.44	0.98	0.00	0.44	0.98
	(0.00, 0.02)	(0.36, 0.52)	(0.94, 0.99)	(0.00, 0.02)	(0.36, 0.52)	(0.94, 0.99)
s_r	0.00	0.51	0.13	0.00	0.51	0.13
	(0.00, 0.15)	(0.46, 0.52)	(0.12, 0.15)	(0.00, 0.15)	(0.46, 0.52)	(0.12, 0.15)
s_L	0.00	0.00	0.03	0.00	0.00	0.03
	(0.00, 0.15)	(0.00, 0.13)	(0.00, 0.07)	(0.00, 0.15)	(0.00, 0.13)	(0.00, 0.07)
s_R	0.00	0.51	0.14	0.00	0.51	0.14
	(0.00, 0.21)	(0.46, 0.52)	(0.12, 0.16)	(0.00, 0.21)	(0.46, 0.52)	(0.12, 0.16)
P value	1.0000	0.9320	0.0877	1.0000	0.9320	0.0877
dLPOD (candidate vs reference)	0.00	0.04	0.02	0.00	0.04	0.02
	(–0.02, 0.02)	(–0.08, 0.15)	(–0.01, 0.06)	(–0.02, 0.02)	(–0.08, 0.15)	(–0.01, 0.06)
dLPOD (candidate presumptive vs candidate confirmed)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)	(–0.02, 0.02)	(–0.10, 0.13)	(–0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

monocytogenes (LMX) assay kit from bioMérieux (595 Anglum Rd, Hazelwood, MO 63042-2330).

(**a**) *VIDAS or miniVIDAS automated immunoassay System.*

(**b**) *LMX reagent strips.*—Sixty polypropylene strips of 10 wells, each strip covered with a foil seal and label. The 10 wells contain the reagents in Table **2013.11C**.

(**c**) *SPR.*—Sixty SPRs coated with proteins specific for *Listeria* receptors.

(**d**) *Standard.*—One vial (1×6 mL). Ready-to-use. Contains

Table 2013.11B. Summary of results for the detection of *Listeria monocytogenes* in queso fresco (125 g)

Method[a]	VIDAS LMX w/OXA			VIDAS LMX w/ALOA		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/total No. samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate presumptive POD (CP)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r[b]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L[c]	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R[d]	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value[e]	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Candidate confirmed positive/total No. samples analyzed	0/144	70/144	144/144	0/144	70/144	144/144
Candidate confirmed POD (CC)	0.00	0.49	1.00	0.00	0.49	1.00
	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)	(0.00, 0.03)	(0.40, 0.57)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9730	1.0000	1.0000	0.9730	1.0000
Positive reference samples/total No. samples analyzed	0/144	69/144	144/144	0/144	69/144	144/144
Reference POD	0.00	0.48	1.00	0.00	0.48	1.00
	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)	(0.00, 0.03)	(0.39, 0.56)	(0.97, 1.00)
s_r	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)	(0.00, 0.16)	(0.46, 0.52)	(0.00, 0.16)
s_L	0.00	0.00	0.00	0.00	0.00	0.00
	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)	(0.00, 0.16)	(0.00, 0.12)	(0.00, 0.16)
s_R	0.00	0.51	0.00	0.00	0.51	0.00
	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)	(0.00, 0.22)	(0.46, 0.52)	(0.00, 0.22)
P value	1.0000	0.9672	1.0000	1.0000	0.9672	1.0000
dLPOD (C vs R)	0.00	0.01	0.00	0.00	0.01	0.00
	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.10, 0.13)	(–0.03, 0.03)
dLPOD (CP vs CC)	0.00	0.00	0.00	0.00	0.00	0.00
	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)	(–0.03, 0.03)	(–0.12, 0.12)	(–0.03, 0.03)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P* value = Homogeneity test of laboratory PODs.

purified and inactivated *L. monocytogenes* receptors + preservative + protein stabilizer.

(**e**) *Positive control solution.*—1 × 3 mL. Contains purified and inactivated *L. monocytogenes* antigen + preservative + protein stabilizer.

(**f**) *Negative control solution.*—1 × 6 mL. Contains Tris-buffered saline (TBS; 150 mmol/l) – Tween pH 7.6 + preservative.

(**g**) *Master lot entry (MLE) card.*—One card providing specifications for the factory master data required to calibrate the test: To read the MLE data, please refer to the Operator's Manual.

(**h**) *Package insert.*

Table 2013.11C. Reagents included in 10-well reagent strip

Wells	Reagents (LMX)
1	Sample well: 0.25 mL of enrichment broth, standard or control
2	Prewash solution (600 µL): TRIS-NaCl (150 mmol/L) – Triton X100 pH 7.6 + preservative
3, 4, 7–9	Wash buffer (600 µL): TRIS-NaCl (150 mmol/L) - Tween pH 7.6 + preservative
5	Conjugate (400 µL): biotin-labeled anti-*Listeria monocytogenes* antibodies + preservative
6	Streptavidin – ALP (400 µL)
10	Reading cuvette with substrate (300 µL): 4-Methyl-umbelliferyl phosphate (0.6 mmol/L) + diethanolamine[a] (DEA; 0.62 mol/L or 6.6%, pH 9.2) + preservative

[a] Irritant reagent: *See* VIDAS LPT package insert for more information.

(**i**) *Pipet*.—Disposable to dispense appropriate volumes.

(**j**) *VIDAS Heat and Go*.—Available from bioMérieux, Inc.

(**k**) *Water bath*.—95–100°C or equivalent system.

(**l**) *Bag with filter*.

(**m**) Smasher™ Blender/Homogenizer available from bioMérieux, Inc., or equivalent.

(**n**) *LMX broth*.—Available from bioMérieux, Inc.

(**o**) *Supplement for LMX broth*.

(**p**) *LPT broth*.—Available from bioMérieux, Inc.

(**q**) *Incubators*.—Capable of maintaining 37 ± 1°C, 35 ± 1°C, and 30 ±1°C.

(**r**) *Diagnostic reagents*.—Necessary for culture confirmation of assays.

(**s**) *ALOA Chromogenic Agar*.—Necessary for cultural confirmation as an alternative to selective agar required by appropriate reference method. Available from bioMérieux, Inc.

(**t**) *Tryptic Soy Agar with yeast additive*.

C. General Instructions

(**a**) Components of the kit are intended for use as integral unit. Do not mix reagents or disposables of different lot numbers.

(**b**) Store VIDAS LMX kits at 2–8°C.

(**c**) Do not freeze reagents.

(**d**) Bring reagents to room temperature before inserting them into the VIDAS instrument.

(**e**) Standard, controls and heated test portions are mixed well before using.

(**f**) Include one positive and one negative control with each group of tests.

(**g**) Return unused components to 2–8°C immediately after use.

(**h**) *See* Safety Precautions in the VIDAS LMX package insert (refer to the following sections in the package insert: Warnings and Precautions and Waste Disposal).

(**i**) Please review the policies recommended by the Centers for Disease Control and Protection (CDC) on dealing with pathogens. http://www.cdc.gov/biosafety/publications/bmb15/index.htm/.

D. Preparation of Test Suspension

(**a**) *Pre-enrichment*.—Pre-enrich test portion using filter Stomacher bags to initiate growth of *L. monocytogenes*. For 25 g test portions, add 225 mL pre-LMX broth brought to room temperature (18–25°C) and 500 µL LMX broth supplement to each test portion and homogenize thoroughly for 2 min. For 125 g test portions, add 375 mL LPT broth brought to room temperature (18–25°C) to each test portion and homogenize thoroughly for 2 min.

(**b**) *25 g Test portions*.—After homogenization, incubate for 26–30 h at 37 ± 1°C.

125 g Test portions.—After homogenization, incubate for 24–30 h at 30 ± 1°C.

After the primary enrichment, transfer a 1 mL aliquot into 10 mL LPT broth brought to room temperature (18–25°C) and incubate for 22–26 h at 30 ± 1°C.

(**c**) After incubation, homogenize samples manually and prepare samples for assay according the following procedures (based on sample size):

125 g Test portions.—No heating is necessary for method performance. Load 0.25 mL of enrichment into the VIDAS LMX reagent strip and perform the VIDAS test.

25 g Test portions.—Follow appropriate instructions based on heating method.

(*1*) *Boiling*.—Transfer 2–3 mL of the enrichment broth into a tube. Seal the tube. Heat in a water bath for 5 ± 1 min at 95–100°C. Cool the tube. Mix the boiled broth and transfer 0.25 mL into the sample well of the VIDAS LMX reagent strip. Perform the VIDAS test.

(*2*) *Heat and Go*.— Transfer 0.25 mL of the enrichment broth into the sample well of the VIDAS LMX reagent strip. Heat for 5 ± 1 min (*see* VIDAS Heat and Go User's Manual). Remove the strip and allow to cool for 10 min prior to test initiation. Perform the VIDAS test.

E. Enzyme Immunoassay

(**a**) Enter factory master calibration curve data into the instrument using the MLE card.

(**b**) Remove the kit reagents and materials from refrigerated storage and allow them to come to room temperature for at least 30 min.

(**c**) Use one VIDAS LMX reagent strip and one VIDAS LMX SPR for each sample, control or standard to be tested. Reseal the storage pouch after removing the required number of SPRs.

(**d**) Enter the appropriate assay information to create a work list. Enter the test code by typing or selecting "LMX", and number of tests to be run. If the standard is to be tested, identify the standard by "S1" and test in duplicate. If the positive control is to be tested, identify it by "C1". If the negative control is to be tested, identify it by "C2".

Note: The standard must be tested upon receipt of a new lot of reagents and then every 14 days. The relative fluorescence value (RFV) of the standard must fall within the set range provided with the kit.

(**e**) Load the LMX reagents strips and SPRs into the positions that correspond to the VIDAS section indicated by the work list. Verify that the color labels with the assay code on the SPRs and reagent strips match.

(**f**) Initiate the assay processing as directed in the VIDAS operator's manual.

(**g**) After the assay is completed, remove the SPRs and reagent strips from the instrument and dispose of properly.

Table 2013.11D. Interpretation of test

Test value threshold	Interpretation
<0.05	Negative
≥0.05	Positive

F. Results and Interpretation

The results are analyzed automatically by the VIDAS system. A report is printed which records the type of test performed, the test sample identification, the date and time, the lot number, and expiration date of the reagent kit being used, and each sample's RFV, test value, and interpreted result (positive or negative). Fluorescence is measured twice in the reagent strip's reading cuvette for each sample tested. The first reading is a background reading of the substrate cuvette before the SPR is introduced into the substrate. The second reading is taken after incubating the substrate with the enzyme remaining on the interior of the SPR. The test value is calculated by the instrument and is equal to the difference between the background reading and the final reading. The calculation appears on the result sheet. A "negative" result has a test value less than the threshold (0.05) and indicates that the sample does not contain *L. monocytogenes* or contains *L. monocytogenes* at a concentration below the detection limit. A "positive" result has a test value equal to or greater than the threshold (≥0.05) and indicates that the sample may be contaminated with *L. monocytogenes.* If the background reading is above a predetermined cutoff, then the result is reported as invalid (Table **2013.11D**).

G. Confirmation

All positive VIDAS LMX results must be culturally confirmed. Confirmation should be performed using the non-heated enrichment broth (the LMX primary enrichment broth for 25 g test portions and the LPT secondary enrichment broth for 125 g test portions) stored between 2–8°C, and should be initiated within 72 h following the end of incubation (AFNOR Certificate No. BIO 12/33-05/12). Presumptive positive results may be confirmed by isolating on selective agar plates such as ALOA or on the appropriate reference method selective agar plates. Typical or suspect colonies from each plate are confirmed as described in appropriate reference method. As an alternative to the conventional confirmation for *L. monocytogenes,* VITEK 2 GP Biochemical Identification (AOAC **2012.02**) or API *Listeria* biochemical kits may be used for presumptive generic identification of foodborne *L. monocytogenes*.

Results of Collaborative Study

In this collaborative study, the VIDAS *Listeria monocytogenes* (LMX) method was compared to the to AOAC **993.12** for one food product, queso fresco, at two test portion sizes, 25 and 125 g. A total of 14 laboratories throughout the United States participated in this study, with 14 laboratories submitting data for the 25 g test portions and 13 laboratories submitting data for the 125 g test portions as presented in Table 1. Each laboratory analyzed 36 test portions for each method 12 inoculated with a high level of *L. monocytogenes,* 12 inoculated with a low level of *L. monocytogenes,* and 12 uninoculated controls. A background screen of the matrix indicated an absence of indigenous *Listeria* species. As per criteria outlined in Appendix J, fractional positive results were obtained for both the 25 and 125 g test portions sizes. For each test portion size, the actual level of *L. monocytogenes* was determined by MPN determination on the day of initiation of analysis. The results of the inoculum heat-stress protocol are presented in Table 2. The individual laboratory and sample results are presented in Tables 3 and 4. Tables **2013.11A** and **B** summarize the collaborative study results for all foods tested, including POD statistical analysis (10). Detailed results for each laboratory are presented in Tables 2A–D, and Figures 1A–D and 2A–D as supplemental data on the *J. AOAC Int.* website.

Queso Fresco (25 g Test Portions)

Queso fresco test portions were inoculated at a low and high level and were analyzed (Table 3) for the detection of *L. monocytogenes*. Uninoculated controls were included in each analysis. Fourteen laboratories participated in the analysis of this matrix and the results of 13 laboratories were included in the statistical analysis. Laboratory 11 reported 11 test portions (including four uninoculated test portions) that produced non-*L. monocytogenes* profiles. Colonies on these plates contained one or more of the following biochemical reactions not typically associated with *L. monocytogenes*: Gram-negative, Gram-positive with spores, non-beta-hemolytic, and catalase negative. Based on the preliminary biochemical tests conducted, the test portions should not have been carried through for final biochemical identification on API *Listeria* strips which resulted in the misidentification of the test portion as *Listeria* spp. The selective agar plates for these test portions were sent to the coordinating laboratory for further examination. The coordinating laboratory confirmed the supplementary results (Gram stain, hemolysis, and catalase reaction) reported by

Table 1. Participation of each collaborating laboratory[a]

	Queso fresco	
Lab	25 g test portions	125 g test portions
1	Y	Y
2	Y[b]	Y
3	Y	Y
4	Y	Y
5	Y	Y
6	Y	Y
7	Y	Y
8	Y	Y
9	Y	Y
10	Y	Y
11	Y[c]	Y[c]
12	Y	Y
13	Y	Y
14	Y	Y

[a] Y= Collaborator analyzed the food type.

[b] Results were not submitted to the coordinating laboratory.

[c] Results were not used in statistical analysis due to laboratory error.

Table 2. Heat-stress injury results

Matrix (LMX test portion size)	Test organism[a]	CFU/OXA (selective agar)	CFU/TSA (nonselective agar)	Degree injury, %
Queso fresco LMX – 25 g	*L. monocytogenes* ATCC 19115	5.0×10^8	1.3×10^9	62
Queso fresco LMX – 125 g	*L. monocytogenes* ATCC 19115	2.9×10^8	9.0×10^8	68

[a] ATCC = American Type Culture Collection.

the participating laboratory and were not able to identify any *Listeria* species. Laboratory 11 also reported one uninoculated control portion positive for *L. monocytogenes*. Testing at the coordinating laboratory verified this result, which indicated cross contamination with that sample. The results from this laboratory were excluded from statistical analysis. The MPN obtained for this matrix, with 95% confidence intervals, were 0.55 CFU/test portion (0.43, 0.70) for the low inoculum level and 3.81 CFU/test portion (3.06, 5.48) for the high inoculum level. For VIDAS LMX test portions, no difference was observed between confirmation of samples using the proprietary chromogenic ALOA agar and OXA required by the reference method.

For the high inoculum level, 156 out of 156 test portions were reported as positive by the VIDAS LMX method with all test portions confirming positive. For the low inoculum level, 77 out of 156 test portions were reported as positive by the VIDAS LMX method with 75 test portions confirming positive, indicating two false-positive results. For the uninoculated controls, 0 out of 156 samples produced a presumptive positive result by the VIDAS LMX method with no samples confirming positive. For test portions analyzed by AOAC **993.12**, 153 out of 156 high inoculum test portions and 69 out of 156 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 156 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.04 (–0.08, 0.15) was obtained between AOAC **993.12** and VIDAS LMX. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.01 (–0.10, 0.13) was obtained between presumptive and confirmed VIDAS LMX results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.02 (–0.01, 0.06) was obtained between AOAC **993.12** and VIDAS LMX. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.00 (–0.02, 0.02) was obtained between presumptive and confirmed VIDAS LMX results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Results of the POD statistical analysis are presented in Table **2013.11A**, Tables 2A–B, and Figures 1A–D as supplemental data on the *J. AOAC Int.* website.

Queso Fresco (125 g Test Portions)

Queso fresco test portions were inoculated at a low and high level and analyzed (Table 4) for the detection of *L. monocytogenes*. Uninoculated controls were included in each sample set. Fourteen laboratories participated in the analysis of this matrix and the results of 12 laboratories were included in the statistical analysis. Laboratory 2 did not report any data for this matrix. Laboratory 11 reported 10 reference method test portions (including five uninoculated control replicates) that produced non-*L. monocytogenes* profiles, with five of the test portions producing questionable API profiles of *L. grayi*. Colonies on these plates contained one or more of the following biochemical reactions not typically associated with *L. monocytogenes*: Gram-negative, non-beta-hemolytic, and catalase negative. Based on the preliminary biochemical tests conducted, the test portions should not have been carried through for final biochemical identification on API *Listeria* strips which resulted in the misidentification of the test portion as *Listeria* spp. The selective agar plates for these test portions were sent to the coordinating laboratory for further examination. The coordinating laboratory verified the supplementary results (Gram stain, hemolysis, and catalase reaction) reported by the participating laboratory and were not able to identify any *Listeria* species. The results from this laboratory were excluded from statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.59 CFU/test portion (0.46, 0.74) for the low level and 5.41 CFU/test portion (3.53, 8.30) for the high level. For VIDAS LMX test portions, no differences were observed between confirmation of samples using the proprietary chromogenic ALOA and the reference method agar.

For the high-level, 144 out of 144 test portions were reported as positive by VIDAS LMX with all test portions confirming positive. For the low level, 70 out of 144 test portions were reported as positive by VIDAS LMX with all 70 test portions confirming positive. For the uninoculated controls, 0 out of 144 samples produced a presumptive positive result by VIDAS LMX and no samples confirmed positive. For test portions analyzed by AOAC **993.12**, 144 out of 144 high inoculum and 69 out of 144 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 144 test portions confirmed positive.

For the low-level inoculum, $dLPOD_C$ values of 0.01 (–0.10, 0.13) were obtained between AOAC **993.12** and VIDAS LMX. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.12, 0.12) were obtained between presumptive and confirmed VIDAS LMX results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high-level inoculum, $dLPOD_C$ values of 0.00 (–0.03, 0.03) were obtained between AOAC **993.12** and VIDAS LMX. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. $dLPOD_{CP}$ values of 0.00 (–0.03, 0.03) were obtained between presumptive and confirmed VIDAS LMX results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table **2013.11B**,

Table 3. Individual collaborator results for queso fresco[a] (25 g test portions)

	VIDAS LMX[b]																																	
	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	–	–[c]	–[c]		–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
11[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[e]	+	–	–	–	+[f]	+	–	–	–	–	–	–	–	–	–	–	–	+[e]	–	–	+[e]
12	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
	AOAC **993.12**																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	–	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	+	+	–	–	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+[g]	+[g]	+	+[h]	+	+	+[i]	+	+	–	+[h]	–	+[h]	–	–	–	+[h]	–	–	+[j]	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Listeria* spp. were detected in samples; – = *Listeria* spp. were not detected in sample.

[b] Confirmed results from OXA and ALOA were identical for each test portion.

[c] Sample was presumptive positive on VIDAS LMX and confirmed negative.

[d] Results were not used in statistical analysis.

[e] Sample presumptive negative on VIDAS LMX, result was reported positive as *L. seeligeri*.

[f] Sample was presumptive positive on VIDAS LMX but result was reported positive as *L. welshimeri*.

[g] Result was reported positive as *L. innocua*.

[h] Result was reported positive as *L. ivanovii*.

[i] Result was reported positive as *L. grayi*.

[j] Sample confirmed positive as *L. monocytogenes*.

Table 4. Individual collaborator results for queso fresco[a] (125 g test portions)

	VIDAS LMX[b]																																	
	High–level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
	AOAC **993.12**																																			
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2[c]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
5	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	+	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
11[c]	+	+	+	+	+	+	+	+	+	+	+	+	+[d]	+	+[e]	+	+	+[f]	+[f]	–	–	+[f]	+	+	–	–	+[f]	+[e]	–	–	–	–	–	+[g]	+[g]	+[g]
12	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Listeria* spp. were detected in samples; – = *Listeria* spp. were not detected in sample.

[b] Results were not used in statistical analysis; NA = not applicable (data not submitted).

[c] Confirmed results from OXA and ALOA were identical for each test portion.

[d] Result reported as *L. welshimeri.*

[e] Result reported as *L. innocua.*

[f] Result reported as *L. grayi.*

[g] Results reported as *L. ivanovii.*

Tables 2C–D, and Figures 2A–D, as supplemental data on the *J. AOAC Int.* website.

ALOA Chromogenic Agar

Confirmatory results obtained from the ALOA chromogenic agar were identical to the results obtained from the OXA agar specified by AOAC **993.12**. Out of a total of 447 positives detected by the VIDAS LMX, 445 were confirmed positive using OXA or ALOA selective agars.

Discussion

No negative feedback was reported to the study directors from the collaborating laboratories in regards to the performance of the VIDAS LMX assay or the ALOA chromogenic agar. Many

laboratories indicated difficulty in identifying and isolating colonies from samples when using OXA plates, but not from test portions analyzed by the VIDAS LMX method. This may be due to the higher selectivity of the ALOA agar to isolate and differentiate typical *Listeria* colonies from competing microflora, such as *Bacillus* colonies. The high selectivity of the proprietary broth, the high background flora and the low selectivity of the OXA agar most likely contributed to this observation as well.

For the analysis of 25 g test portions by the VIDAS LMX method, two false positives were obtained. The test results produced by the false-positive test portions (average test value of 0.22) were much lower than the test values observed with true positives (average value >2.00). By the time the coordinating laboratory received the results, the primary enrichments for these samples had been discarded so no subsequent analysis on the VIDAS LMX was possible. However, the agar plates for these test portions were shipped to the coordinating laboratory for further analysis. Up to 20 different colonies were picked for morphological and biochemical analysis using VITEK 2 GP and no *Listeria* colonies were identified. Additionally, the entire lawn of growth from each agar plate was swabbed, enriched in LMX broth, and incubated for 26–30 h at 37 ± 1°C. An aliquot from each tube was analyzed by the VIDAS LMX assay and negative results for *L. monocytogenes* were obtained. Results of this investigation led the study directors to believe that the false positives were the result of possible cross-contamination during the analysis of the samples.

For the analysis of both the 25 and 125 g test portions, Laboratory 11 detected the presence of multiple types of *Listeria* spp. An investigation into the results indicated that colonies picked for confirmation did not meet the characteristics of *Listeria* spp., i.e. colonies produced Gram-negative stain reactions and were negative for motility and catalase. The results of these tests should have precluded analysis using the API strips which lead to an inaccurate identification. Due to the fact that final results reported were inconsistent with biochemical results, data produced by Laboratory 11 was removed from the statistical analysis of both the 25 and 125 g test portions.

Using the POD statistical model, no significant difference in the number of positive results obtained between the two methods being compared was observed at both the low and high inoculum levels for both the 25 and 125 g test portions. No significant difference was observed between presumptive and confirmed results for the candidate method.

Conclusions

The VIDAS *Listeria monocytogenes* Xpress (LMX) method with the optional ALOA agar confirmation method was adopted as Official First Action status for the detection of *L. monocytogenes* in a variety of foods, including deli ham (25 and 125 g), fermented sausage (25 g), liver pâté (25 g), processed cheese (25 g), vanilla ice cream (25 g), cooked shrimp (25 g), smoked white fish (25 g), frozen spinach (25 g), peanut butter (25 g), deli turkey (25 and 125 g), queso fresco (125 g), and ground beef (125 g).

Acknowledgments

We would like to extend a sincere thank you to the following collaborators for their dedicated participation in this study:

John Mills and Pat Rule, bioMérieux Industry (Hazelwood, MO)

Ben Howard, Neil Rogman, and Jacob Cannon, Certified Laboratories (Bollingbrook, IL)

Barbara Paul, Marianna Sala-Rhatigan, and Susan Joseph, U.S. Food and Drug Administration, Northeast Regional Laboratory (Jamaica, NY)

Nikki Palen, Amber Stegmann, and Bryan Perry, EMS Laboratories (St. Louis, MO)

Rachel Hiles and Tenesha Stubblefield, Silliker Laboratories (Omaha, NE)

Nigel Nagassar, Sylvanus Owusu, and Jacqueline Zimmerman, Silliker Laboratories (Minnetoka, MN)

Jerry Lynn Pickett, Jacquelyn Adams, Aaron Bollenbacher, Keith Wiggins, and Lori Cesanas-Tyson, WBA Analytical (Springdale, AR)

Bharath Brahmanda, Food Safety Net Services (San Antonio, TX)

Andrew Capps, Grisel Rosario, Dawn Davis, Lindsey Parker, Christine Said, and Jianfeng Li, North Carolina Department of Agriculture and CS: Food and Drug Protection Division (Raleigh, NC)

Keith Klemms, Bill May, Becky Hand, and Rose Burkhart, Sherry Laboratories (Warsaw, IN)

Hesham Elgaali, Indiana State Department of Health, Food and Dairy Microbiology Division (Indianapolis, IN)

Jennifer Jolly, Covance Laboratories (Battle Creek, MI)

Sandy Moore, Dustin Ebbing, Maggie Michels, Amanda Kehres, and Joe Hirsch, John Morrell & Co. (Springdale, OH)

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FOOD BIOLOGICAL CONTAMINANTS

Evaluation of the ANSR® for *Salmonella* Assay for Identification of *Salmonella* spp. from Colony Picks from Selective/Differential Agar Media: First Action 2013.14

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A collaborative study was conducted to evaluate performance of the ANSR® for *Salmonella* assay for identification of *Salmonella* spp. from colony picks taken from selective/differential agar media. The ANSR *Salmonella* assay is an isothermal nucleic acid amplification test based on the nicking enzyme amplification reaction chemistry. The test can be completed in less than 40 min including sample preparation. A total of 18 laboratories representing industry, government, academic, and commercial testing laboratories participated in the study. Each collaborator tested up to 84 samples, comprised of colony picks of six *Salmonella* spp. and six non-salmonellae taken from six selective/differential agar media as well as tryptic soy agar. A total of 1441 analyses were performed, 1416 of which gave the correct identification, for overall accuracy of 98.3%. For identification of *Salmonella* spp., 755 of 756 tests (99.9%) produced the correct result. For identification of non-salmonellae as such, 661 of 685 assays (96.5%) produced the correct result. Of the 18 laboratories, 15 produced data sets with 99–100% accuracy. The majority of false-positive results were clustered in three laboratories; analysis of raw data suggests procedural difficulties in at least two cases, which may explain the atypical data from these collaborators. The ANSR *Salmonella* assay can be used as a rapid, accurate adjunct or alternative to biochemical testing for identification of presumptive *Salmonella* spp. isolates.

Received December 18, 2013.
The method was approved by the Expert Review Panel for Food Biological Contaminants as First Action.
The Expert Review Panel for Food Biological Contaminants invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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An appendix is available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI: 10.5740/jaoacint.14-004

Identification of presumptive *Salmonella* colonies from selective/differential agar media as *Salmonella* spp. has historically been achieved using a variety of biochemical and serological procedures. In the case of food and environmental sample analysis, these procedures are specified in reference methods such as those in the U.S. Food and Drug Administration's *Bacteriological Analytical Manual* (BAM; 1) and the U.S. Department of Agriculture's *Microbiology Laboratory Guidebook* (MLG; 2). These methods include conventional biochemical tests, miniaturized biochemical test devices, automated biochemical identification platforms, and serological agglutination tests using *Salmonella*-specific antisera. The biochemical identification procedures, although accurate and reliable, generally require 6–24 h to obtain results. The serological procedures may be rapid, but often require subculture to enhance antigen expression, especially in the case of flagellar (H) antigen typing.

As an adjunct or alternative to biochemical and serological procedures, nucleic acid-based identification methods hold promise for providing timely and accurate results. This has been acknowledged, for example, by reference in both BAM and MLG to use of nucleic acid-based methods for identification of *Listeria monocytogenes* (3, 4).

The ANSR® *Salmonella* assay was originally developed for rapid screening of enriched food and environmental samples. The assay is an isothermal nucleic acid amplification procedure, based on the nicking enzyme amplification reaction (NEAR) technology (5). The ANSR method has been evaluated in three AOAC *Performance Tested Method*SM (PTM) validation studies, leading to certification as PTM 061203, with claims for a variety of food and environmental sample types (6–7). In these studies, the ANSR method exhibited sensitivity comparable to that of the BAM and MLG reference culture methods by probability of detection statistical analysis, as well as >99% inclusivity and 100% exclusivity in testing of target and nontarget bacteria.

This method performance, coupled with the simplicity and rapidity of the assay (less than 40 min), suggested that the method could also serve as a useful tool for identification of presumptive *Salmonella* spp. isolates from selective/differential agar plating media. A precollaborative study has been completed in which colonies of 113 *Salmonella* spp. strains and 37 non-*Salmonella* strains were picked from tryptic soy agar (TSA) and six selective/differential agar media [Hektoen enteric agar (HE), xylose lysine

Table 1. Inclusive and exclusive isolates used in the ANSR *Salmonella* collaborative study

Organism	ID No.	Source	Origin (if known)
Salmonella enterica subsp. *arizonae*	700156	ATCC[a]	Poult
Salmonella enterica subsp. *enterica* Ser. Typhimurium	23566	ATCC	Unknown
Salmonella enterica subsp. *enterica* Ser. Cubana	12007	ATCC	Unknown
Salmonella bongori	43975	ATCC	Unknown
Salmonella enterica subsp. *enterica* Ser. Cerro	10723	ATCC	Unknown
Salmonella enterica subsp. *enterica* Ser. Enteritidis	4931	ATCC	Human GI tract
Enterobacter cloacae	13047	ATCC	Human CSF
Escherichia coli	25922	ATCC	Human
Proteus vulgaris	29905	ATCC	Unknown
Providencia alcalifaciens	27970	ATCC	Feces
Citrobacter freundii	8090	ATCC	Unknown
Klebsiella pneumoniae	13883	ATCC	Unknown

[a] American Type Culture Collection, Manassas, VA.

deoxycholate agar (XLD), bismuth sulfite agar (BS), brilliant green sulfa agar (BGS), xylose lysine tergitol agar (XLT-4), and double-modified lysine iron agar (DMLIA)] and tested in the ANSR assay. The former three media are specified for use in the BAM reference method, while the latter three are specified in the MLG method. One hundred and twelve *Salmonella* spp. strains produced positive results from all seven media, for inclusivity of 99.1%. One strain of *S.* Weslaco, previously identified as a non-inclusive strain lacking the genetic target for the ANSR assay, produced negative results from all seven media. In testing of exclusive strains, 248 of 251 assays produced negative results, for accuracy of 98.8%. The precollaborative study report is included as Appendix I on *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac.

Here we report results of an interlaboratory collaborative study conducted in 18 laboratories for further evaluation of the assay as a colony confirmation tool.

Collaborative Study

Study Design

This collaborative study was conducted in accordance with the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces, Appendix J* (8). Eighteen laboratories participated in the collaborative study, representing industry, academic, government, and private testing laboratories. All collaborators were either established users of the ANSR test system or were expressly trained for the collaborative study prior to its commencement. A detailed set of instructions and data recording forms were sent to each collaborator in advance of the study. Collaborators were provided with all necessary agar plating media, test kits, ANSR system instrumentation, and a blind-coded set of 12 bacterial cultures for analysis.

Preparation of Isolates

All isolates were from the Neogen Corp. culture collection and consisted of six diverse strains of *S. enterica* and *S. bongori*, and six strains of Enterobacteriaceae belonging to other genera (Table 1). All strains were obtained directly from the American Type Culture Collection (ATCC; Manassas, VA). Identity of isolates was confirmed by API 20E testing. *Salmonella* isolates were also verified by O group serology. Isolates were cultured on TSA slants for 18–24 h at 36 ± 1°C. Slant cultures were labeled with a two-digit alphabetical code.

Distribution of Isolates

Cultures were shipped to collaborators via overnight delivery, at ambient temperature, using Category B Dangerous Goods packaging as set forth by International Air Transport Association regulations. Collaborators were instructed to store the cultures at 2–8°C until initiation of the analytical work (4–5 days). Collaborators were provided with a "Sample Receipt Form," to be completed and returned to the Study Director by email or fax, acknowledging that the samples were received in good condition.

Analysis of Isolates

To initiate the analysis, collaborators streaked each of the 12 bacterial isolates to each of the seven agar media, streaking for isolated colonies. Collaborators were provided with a sample randomization scheme by the Study Director and were instructed to blind-code each strain-agar medium combination with a unique number 1–84. This was performed by "Operator 1," who would have no involvement in the actual ANSR analyses. Plates were incubated for 24±2 h at 35±1°C and examined for the presence of isolated colonies. Plates without isolated colonies were reincubated for an additional 18–24 h. Plates containing distinct isolated colonies after 24 h were stored at 2–8°C. After a maximum of 48 h incubation, plates without growth or isolated colonies were noted as such on the Data Recording Form and analysis continued. Operator 1 then picked a single colony from each plate, including the refrigerated plates, using an inoculating loop or needle, and resuspended the colony in 0.5 mL phosphate-buffered saline (PBS). The coded tubes were transferred to "Operator 2," who then performed the ANSR analyses. ANSR testing was performed in blocks of up to 16 samples, starting with sample number 1 and continuing through sample number

84. Completed Data Recording Forms were returned to the Study Director by email or fax. ANSR assay raw data were provided to the Study Director by email as .json files. This raw data included the real-time fluorescence curves for each assay performed.

AOAC Official Method 2013.14
Identification of *Salmonella* spp. from Colony Picks
ANSR® *Salmonella* Confirmation Test
First Action 2013

(Applicable to the identification of *Salmonella* spp. from colony picks from selective/differential agar media: Bismuth sulfite agar, brilliant green sulfa agar, double-modified lysine iron agar, Hektoen enteric agar, tryptic soy agar, xylose lysine deoxycholate agar, and xylose lysine tergitol agar.)

See Tables **2013.14A** and **B** for a summary of results of the collaborative study.

Safety precautions.—Use of this test should be restricted to individuals with appropriate laboratory training in microbiology and molecular techniques. Reagents are for laboratory use only. Refer to the Material Safety Data Sheet from Neogen Corp. for more information. Enrichment cultures, used agar plates, and ANSR assay lysates and reaction tubes should be handled and disposed of as potentially infectious material and Biosafety Level 2 measures employed. The preferred method for disposal of contaminated materials, including cultures, pipet tips, tubes, etc., is autoclaving. Items that cannot be autoclaved should be decontaminated by treatment with disinfectant solution. ANSR reaction tubes should not be autoclaved in areas where they may open and possibly contaminate the laboratory environment with amplification products. Alternatively, they may be disposed of in a sealed container with a small amount of 10% household bleach added.

A. Principle

ANSR *Salmonella* is an isothermal nucleic acid amplification assay based on the nicking enzyme amplification reaction (NEAR) technology (5). The amplification mechanism involves binding of an oligonucleotide "template" to a specific sequence of target DNA. The template contains a recognition site for a specific endonuclease. The nicked strand is recognized as damaged and repaired by the action of a thermostable DNA polymerase, displacing the original strand with the newly-synthesized repaired portion. This displaced DNA "product" then binds to a second template and the same reactions lead to formation of a second product. Amplification products are detected using a specific molecular beacon probe. Fluorescent signal is generated in real time, with amplification and detection complete within 10 min. The entire assay is conducted at a constant temperature of 56°C using a temperature-controlled fluorescence detection instrument. Assay software analyzes the fluorescent signal over time; a data interpretation algorithm interprets results as negative, positive, or invalid based on baseline, rate-of-change, and other criteria. Each tube of ANSR reagents also contains an internal positive control, signaling in a second fluorescence channel irrespective of the presence of target DNA, and indicating proper functioning of the amplification reagents.

Table 2013.14A. Interlaboratory study results for the ANSR *Salmonella* test: Inclusive isolates

Organism	Correct	Misidentified	Total
Salmonella enterica subsp. *arizonae*	126	0	126
Salmonella enterica subsp. *enterica* Ser. Typhimurium	126	0	126
Salmonella enterica subsp. *enterica* Ser. Cubana	126	0	126
Salmonella bongori	126	0	126
Salmonella enterica subsp. *enterica* Ser. Cerro	126	0	126
Salmonella enterica subsp. *enterica* Ser. Enteritidis	125	1	126
Total isolates	755	1	756

B. Media and Reagents

(**a**) *ANSR® for Salmonella test kit.*—Available from Neogen Corp., Cat. No. 9843 (Lansing, MI, www.neogen.com). Contains: Lyophilized reagents in capped strip tubes, eight tubes per strip, 12 strips (96 tests) per kit, in two sealed foil pouches with desiccant packs; cluster tubes, eight tubes per strip, 12 strips per kit; permanent caps, eight caps per strip, 12 strips per kit; lysis buffer, one bottle, 60 mL; lysis reagent, three vials, lyophilized; kit insert. Store reagent tubes at 2–8°C, in sealed foil pouches with desiccant. Store lysis buffer at 2–8°C.

(**b**) *Phosphate-buffered saline (PBS).*—Per liter: 8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4.

(**c**) *Hektoen enteric agar (HE).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**d**) *Xylose lysine deoxycholate agar (XLD).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**e**) *Bismuth sulfite agar (BS).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**f**) *Brilliant green sulfa agar (BGS).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**g**) *Xylose lysine tergitol agar (XLT-4).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

(**h**) *Double-modified lysine iron agar (DMLIA).*—Available

Table 2013.14B. Interlaboratory study results for the ANSR *Salmonella* test: Exclusive isolates

Organism	Correct	Misidentified	Total
Enterobacter cloacae	96	2	98
Escherichia coli	117	8	125
Proteus vulgaris	102	4	106
Providencia alcalifaciens	105	2	107
Citrobacter freundii	122	4	126
Klebsiella pneumoniae	119	4	123
Total isolates	661	24	685

from various suppliers. Follow manufacturer's instructions for preparation.

(**i**) *Tryptic soy agar (TSA).*—Available from Neogen Corp. and other suppliers. Follow manufacturer's instructions for preparation.

C. Apparatus

(**a**) *Incubator/reader.*—Available from Neogen Corp. Incubator/reader capable of operating at 56 ± 1°C and reading fluorescence in real time in two channels (485/535 nm and 540/590 nm).

(**b**) *Computer and ANSR software.*—Available from Neogen Corp. For connection to incubator/reader. Minimum requirements for computer: Intel® Core i3 processor, 1 GB RAM, Windows® 7, Ethernet, and USB connections.

(**c**) *Heater block.*—With insert for 1.2 mL cluster tubes, 80 ± 2°C.

(**d**) *Micropipettor.*—50 µL, fixed or adjustable volume.

(**e**) *Pipettor.*—100–1000 µL, adjustable volume.

(**f**) *8-Channel micropipettor.*—20–200 µL, adjustable volume.

(**g**) *Pipet tips.*—100 µL, with filter.

(**h**) *Pipet tips.*—1000 µL.

(**i**) *Tubes.*—Glass or plastic, 12 × 75 mm or similar, sterile, with caps.

(**j**) *Inoculating loops or needles.*—Sterile.

D. Preparation of Test Samples

Pick an isolated colony from nonselective or selective/differential agar medium (one of the media listed in section **B**) with an inoculating loop or needle and resuspend (vortex or otherwise thoroughly mix) in 0.5 mL PBS in a sterile, capped tube.

E. Test Procedure

(**a**) *General preparation.*—(*1*) This assay should be performed in a controlled laboratory environment.

(*2*) Do not use culture media or ANSR reagents beyond their expiration dates. Do not interchange reagents between ANSR kit lots.

(*3*) Remove ANSR reaction tubes from the foil pouch just before use. Avoid prolonged exposure to light. Tap reaction tubes on bench top to make sure that lyophilized reagents are at the bottom of the tube prior to adding the lysed sample.

(*4*) Complete all assay steps in sequence, avoiding delays between steps.

(*5*) Exercise care in pipetting steps to avoid cross-contamination of samples.

(*6*) Do not remove caps from reaction tubes at any point after the assay is started; this will prevent accidental contamination of the environment with amplification products.

(*7*) *Prior to starting the assay.*—(*i*) Preheat the lysis heater block to 80 ± 2°C. (*ii*) Start the ANSR software using the computer connected to the ANSR reader. Select "*Salmonella*" as the test type. Enter sample identifications and other experiment information. The reader will preheat to 56 ± 1°C.

(**b**) *Assay procedure.*—(*1*) Add 50 µL of colony resuspension to a 1.2 mL cluster tube. Use a new pipet tip for each sample.

(*2*) Add 450 µL lysis buffer to the cluster tube. *Note*: It is not necessary to use the lysis reagent provided with the test kit for this application.

(*3*) Transfer the cluster tubes to the 80°C heater block and incubate for 20 min. *Note:* The incubation time may be extended to a maximum of 60 min for the purpose of managing staggered assay start times.

(*4*) Approximately 3 min before the end of the lysis step, preheat the ANSR reaction tubes to 56°C by placing the tubes in the incubator/reader. *Note:* The strip of tubes may be cut to provide the number of tubes needed.

(*5*) At the end of the 20 min lysis incubation, remove and discard the caps from the reaction tubes.

Note: Steps (*6*)–(*8*) should be completed without delay (within 1 min).

(*6*) Using an 8-channel micropipettor and 100 µL tips with filters, carefully transfer 50 µL of the lysed samples to the reaction tubes. Mix by rapidly pipetting up and down at least 10 times until the sample appears homogenous in the pipet tip. Avoid excessive bubble formation by not depressing the pipettor plunger beyond the first stop.

(*7*) Place the permanent caps on the reaction tubes and close the lid of the incubator/reader.

(*8*) Click START in the ANSR software to begin the assay.

(*9*) The assay will complete in 10 min and results will be displayed.

F. Interpretation of Results

The ANSR software will indicate the test results as POSITIVE, NEGATIVE, or INVALID. A positive result indicates that the colony tested contains *Salmonella* spp. A negative result indicates that the colony tested does not contain *Salmonella* spp. Assays producing invalid results must be repeated. The real-time fluorescence curves for both the test and positive control channel can be viewed using the ANSR software.

G. Limitations

The assay detects serovars of both *S. enterica* and *S. bongori*, including all genetic subgroups. In testing of 113 strains of *Salmonella* spp., representing 108 serovars, only a single strain of *S.* Weslaco was not detected.

Results

A summary of results for inclusive and exclusive isolates is shown in Tables **2013.14A** and **B**, respectively. Detailed results, by collaborating laboratory, are shown in Tables 2 and 3. For inclusive strains, all collaborators reported that all six strains grew on all media and a total of 756 ANSR analyses were performed. There were 755 positive results, for accuracy of 99.9% in identification of presumptive *Salmonella* spp. colonies. Laboratory 2 reported a negative result for *S.* Enteritidis on HE agar. There is no obvious explanation for this result.

There were a maximum of 756 possible results on exclusive strains. There were 65 cases of reported no growth or lack of distinct isolated colonies. A detailed analysis of results showed that three collaborators (laboratories 3, 12, and 13) reported no growth for the *Enterobacter cloacae* culture on all seven media, accounting for 21 of the no-growth results. Most collaborators reported that neither *Providencia alcalifaciens* nor *Proteus*

Table 2. Inclusivity panel results[a]

Organism	Culture medium[b]	Collaborating laboratory																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
S. enterica subsp. *arizonae*	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. enterica Ser. Typhimurium	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. enterica Ser. Cubana	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. bongori	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. enterica Ser. Cerro	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
S. enterica Ser. Enteritidis	BGS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	BS	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	DMLIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	HE	+	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLD	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	XLT-4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a] + = Correctly identified as *Salmonella* spp.; – = incorrectly identified.

[b] BGS = brilliant green sulfa agar; BS = bismuth sulfite agar; DMLIA = double-modified lysine iron agar; HE = Hektoen enteric agar; TSA = tryptic soy agar; XLD = xylose lysine deoxycholate agar; XLT-4 = xylose lysine tergitol agar.

Table 3. Exclusivity panel results[a]

Organism	Culture medium[b]	Collaborating laboratory																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Enterobacter cloacae	BGS	–	–	NG[c]	–	–	–	–	–	–	–	+	NG	NG	–	–	+[d]	–	–
	BS	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	DMLIA	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	HE	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	TSA	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	XLD	–	+	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	+[d]	–	–
	XLT-4	–	–	NG	–	–	–	–	–	–	–	–	NG	NG	–	–	NG	–	–
Escherichia coli	BGS	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–
	BS	–	–	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–
	DMLIA	–	+	–	–	–	–	–	–	–	–	–	–	–	NG	–	–	–	–
	HE	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	TSA	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLD	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLT-4	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Proteus vulgaris	BGS	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	–	–	+
	BS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	DMLIA	–	–	–	–	–	–	–	–	NG	–	–	–	–	NG	–	–	–	–
	HE	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	TSA	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–
	XLD	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLT-4	NG	NG	–	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Providencia alcalifaciens	BGS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	BS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
	DMLIA	–	–	–	NG	–	–	–	–	NG	–	–	NG	–	NG	–	–	–	–
	HE	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	TSA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLD	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLT-4	–	NG	–	NG	NG	NG	–	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
Citrobacter freundii	BGS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	BS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	DMLIA	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	–	–	–
	HE	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	TSA	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLD	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	XLT-4	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Klebsiella pneumoniae	BGS	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	BS	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	–	–	–
	DMLIA	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	–	–	–
	HE	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	TSA	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	XLD	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
	XLT-4	–	–	–	–	–	–	–	–	–	–	–	–	–	NG	–	+	–	–

[a] – = Correctly identified as not *Salmonella* spp.; + = incorrectly identified.

[b] BGS = brilliant green sulfa agar; BS = bismuth sulfite agar; DMLIA = double-modified lysine iron agar; HE = Hektoen enteric agar; TSA = tryptic soy agar; XLD = xylose lysine deoxycholate agar; XLT-4 = xylose lysine tergitol agar.

[c] No growth or no isolated colonies on plate.

[d] Suspected contaminated culture. Data removed from statistical analysis.

Table 4. Results by agar medium

Medium[a]		Correct	Misidentified	Total
BGS	Inclusive	108	0	108
	Exclusive	98	5	103
BS	Inclusive	108	0	108
	Exclusive	99	4	103
DMLIA	Inclusive	108	0	108
	Exclusive	93	3	96
HE	Inclusive	107	1	108
	Exclusive	100	4	104
TSA	Inclusive	108	0	108
	Exclusive	101	3	104
XLD	Inclusive	108	0	108
	Exclusive	101	3	104
XLT-4	Inclusive	108	0	108
	Exclusive	69	2	71
Total	Inclusive	755	1	756
	Exclusive	661	24	685

[a] BGS = brilliant green sulfa agar; BS = bismuth sulfite agar; DMLIA = double-modified lysine iron agar; HE = Hektoen enteric agar; TSA = tryptic soy agar; XLD = xylose lysine deoxycholate agar; XLT-4 = xylose lysine tergitol agar.

vulgaris produced colonies on XLT-4 agar. The remaining cases of no growth appeared to be random with respect to strain and medium. A total of 691 analyses were performed on exclusive strains. Collaborator 16 reported positive results on six of seven plates streaked with the *E. cloacae* culture. The remaining agar, TSA, was reported to have no growth. Collaborator 16 reported that the six plates all contained growth with colonies of a *Salmonella*-like appearance. It is concluded that this culture became contaminated at some point during preparation or analysis and therefore these data were eliminated from the statistical analysis. Of 685 remaining analyses, 661 produced negative results for accuracy with exclusive strains of 96.5%.

A summary of results by agar medium is shown in Table 4. The percentage of correct results was very similar for all seven media, ranging from 97.6 to 98.9%.

Discussion

In this multilaboratory evaluation of the ANSR *Salmonella* test for identification of presumptive *Salmonella* spp. isolates from agar media, the method exhibited exceptional accuracy with inclusive strains and a high degree of exclusivity with non-salmonellae. Of the 18 laboratories participating in the study, 15 reported results with overall accuracy of 99 to 100%. There was only a single false-negative result out of 756 *Salmonella* spp. colonies tested. Excluding data generated from a suspected contaminated slant culture, there were 24 false-positive results on non-*Salmonella* spp. colonies out of 685 colonies tested. All but seven of these aberrant results occurred in three laboratories. Laboratory 16 reported six false-positive results in addition to those linked to the contaminated slant culture. No further information is available for these samples, except that all six ANSR fluorescence curves were very strong, typical of true positive results. Laboratory 2 reported six false-positive results; four of these occurred in a single ANSR assay run of 15 samples. All but one of the false-positive results showed atypical, weak fluorescence curves, suggestive of cross-contamination during performance of the ANSR assay. Laboratory 13 reported five false-positive results. Again, all but one of these results showed atypical, weak fluorescence curves. Additionally, raw data received from this laboratory indicated that one assay run was repeated in total due to extreme aberrant results (i.e., invalid assays), suggesting that the technician was experiencing difficulty in performing the assay correctly.

Including data from all 18 laboratories (with the exclusion of the six suspected contaminated samples from laboratory 16), accuracy on inclusive and exclusive strains was 99.9 and 96.5%, respectively. Considering only data from the 15 laboratories without clusters of aberrant results, accuracy on exclusive strains was 98.8%.

Recommendations

The ANSR *Salmonella* test was adopted as Official First Action status for use as a rapid, accurate adjunct or alternative to biochemical testing for identification of presumptive *Salmonella* spp. isolates.

Acknowledgments

We thank the following collaborators for their participation in this study:

Dorn Clark and Hondo Dammann, Marshfield Food Safety (Marshfield, WI)

Jessica Dyszel and Matthew Vross, Richter International (Columbus, OH)

Nicole Cuthbert and Brian Kupski, Silliker (Crete, IL)

Joe Benzinger, Megan Boyle, and Jonathan Flannery, Q Laboratories (Cincinnati, OH)

Eric S. Adams, John B. Barrett, Mark E. Berrang, Douglas E. Cosby, Nelson A. Cox, Jonathan G. Frye, Lari M. Hiott, Charlene R. Jackson, Steven W. Knapp, and Luanne L. Rigsby, U.S. Department of Agriculture, Agricultural Research Service (Athens, GA)

Robert Fuller and Jarrod Van Brunt, Tyson Foods (Springdale, AR)

Hamoud Alnughaymishi and Andrew Scollon, Michigan State University, Department of Food Science and Human Nutrition (East Lansing, MI)

Melanie Corebello and Erika Sai, Unilever U.S. (Englewood Cliffs, NJ)

Lisa Kuepfer and Jill Stepnitz, Covance (Battle Creek, MI)

Michael Hudgens and Walter Jones, NP Analytical Laboratories (St. Louis, MO)

Douglas Waltman and Selena York, Georgia Poultry Laboratory (Oakwood, GA)

Jake Cannon, Benjamin Howard, and Neil Rogman, Certified Laboratories of the Midwest (Bolingbrook, IL)

Chad Pidgeon and Amy Quenneville, Ben & Jerry's (Burlington, VT)

Vikas Gill and Hua Wang, United States Food and Drug Administration, Center for Food Safety and Applied Nutrition (College Park, MD)

Cori Flores and Priyanwada Kulkarni, Henningsen Foods (Omaha, NE)

Aaron Copeland and Gordon Whitbeck, Whitbeck Laboratories (Springdale, AR)

Robert Fuller and Melissa Sisemore, WBA Laboratories (Springdale, AR)

Emily Feldpausch and Lei Zhang, Neogen Corp. Research and Development (Lansing, MI)

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