2017 AOAC OFFICIAL METHODS BOARD AWARDS

2014 - 2016 SPIFAN METHODS TO BE REVIEWED FOR

2017 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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SPIFAN METHODS REVIEWED 2014 - 2016

OFFICIAL METHODS

Determination of Biotin by Liquid Chromatography Coupled with Immunoaffinity Column Cleanup Extraction: Single-Laboratory Validation, First Action 2016.02

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Adopted as a First Action *Official Method*SM by the Expert Review Panel on Biotin.
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Introduction

The AsureQuality Auckland Laboratory has initiated a method to facilitate a specific, precise, accurate, and robust procedure for the analysis of biotin from infant formula and adult/pediatric nutritional formulas (1-8). The method also has an assured limit of quantification of 0.1 µg/100 g (1 part per billion; ppb) based on a simple mathematical relationship between lowest standard and dilution. The method involves an immunoaffinity column (R-Biopharm Rhone, EASI-EXTRACT biotin column or equivalent) cleanup and extraction followed by LC–UV set at 200 nm.

AOAC Official Method 2016.02
Biotin
Liquid Chromatography Coupled with Immunoaffinity Column Cleanup Extraction
First Action 2016

A. Principle/Methodology

The sample is dispersed in phosphate-buffered saline (PBS) and autoclaved at 121 ± 2°C for 25 min. The sample is cooled to room temperature and then diluted to 100 mL in a volumetric flask. The extract is centrifuged and filtered using Whatman glass microfiber filter paper (GE Healthcare Life Sciences, Buckinghamshire, UK). Clear filtrate is collected for cleanup and extraction. The biotin immunoaffinity column is mounted onto an SPE manifold. A disposable syringe barrel is connected to the immunoaffinity column as a reservoir. The buffer in the affinity column is drained and the sample filtrate is loaded through the reservoir and allowed to flow through by gravity. The column is washed with PBS followed by water. Air is passed through the column to remove residual liquid.

Biotin from the column is eluted with methanol and collected in a Reacti-Vial (Cat. No. 13223, Thermo Scientific). The eluent is evaporated to dryness using a heating block set at 85 ± 5°C under a gentle stream of nitrogen, and the sample is reconstituted in 1 mL water. The biotin in the reconstituted sample is quantified by HPLC–UV set at 200 nm.

B. Chemicals

(a) *Laboratory reagent-grade water.*
(b) *Sodium dihydrogen phosphate dihydrate.*
(c) *Disodium hydrogen phosphate dihydrate.*
(d) *Sodium hydroxide.*
(e) *Methanol.*—HPLC grade.

(f) *Acetonitrile.*—HPLC grade.

(g) *Orthophosphoric acid.*—85%.

(h) *PBS.*—pH 7.4 (Cat. No 10010031, Life Technologies/Thermo Scientific or equivalent).

(i) *Biotin.*—Purity ≥ 99% (Cat. No. B4501, Sigma Chemical Co; St. Louis, MO or equivalent).

C. Reagents

(a) *Sodium hydroxide, 2 M.*—Weigh 80 g sodium hydroxide in a 1 L volumetric flask, then dissolve in water and make up to the mark.

(b) *Sodium phosphate buffer, 0.15 M.*—Weigh 9.15 g sodium dihydrogen phosphate dihydrate and 16.31 g disodium hydrogen phosphate dihydrate in a 1 L volumetric flask, then dissolve in water and make up to the mark. Adjust the pH to 7 with 2 M sodium hydroxide.

(c) *Phosphoric acid, 0.1%.*—In a 1 L volumetric flask, add 500 mL water. Add 1.2 mL orthophosphoric acid. Mix and make up to the mark with water.

D. Apparatus

(a) *Whatman glass microfiber filters.*—Cat. No. 1820-125.

(b) *R-Biopharm immunoaffinity column pack.*—P82/P82B or equivalent.

(c) *SPE manifold.*—With accessories.

(d) *Autoclave.*—Set at 121°C.

(e) *Centrifuge.*—Variable speed.

(f) *Analytical balance.*—4 dp.

(g) *Amber glass screw-cap bottle.*—100 mL.

(h) *Horizontal shaker.*

(i) *Volumetric flasks.*—1 L and 250, 100, and 10 mL.

(j) *Pipettors.*—Calibrated; 10.0, 5.0, 1.0 mL and 200, 100, and 50 µL.

(k) *Measuring cylinder.*—100 and 50 mL.

(l) *Reacti-Vials.*

(m) *Reacti-Therm heating block.*—With nitrogen blow down (Thermo Scientific).

(n) *Ultrasonic bath.*—Set at 50°C.

(o) *Centrifuge tubes.*—50 mL.

(p) *Vortex mixer.*

(q) *Syringe filter.*—PTFE, 0.45 µm (Cat. No 13HP045AN, Advantec Syringe Filters, Cole-Parmer, IL).

(r) *Disposable syringes.*—10 and 1 mL.

(s) *HPLC vials.*—2 mL with 200 µL glass inserts.

E. Sample Preparation

Note: For weight and loading volumes for the different ranges of product, *see* Table **2016.02A**. Slurry may be used wherever product heterogeneity is expected.

For the slurry, reconstitute the 25 g powder with warm water (~50°C) to a total weight of 200 g. Mix thoroughly on a horizontal shaker for 15 min and then sonicate at 50°C for 10 min. Cool to room temperature.

(a) Weigh sample/slurry into a 100 mL amber glass screw-cap bottle. *See* Table **2016.02A**.

(b) Add 0.15 M sodium phosphate buffer to a volume of 50 mL.

(c) Swirl gently to mix.

(d) Autoclave the sample preparation at 121°C for 25 min.

Table 2016.02A. Sample preparation

Product, µg/100 g		Sample preparation				Concn, µg/100 mL)	
Min	Max	Weight, g	Volume, mL	Load, mL	Final	Min	Max
0.1	0.5	20	100	50	1 mL	1	5
0.5	1.0	10	100	20	1 mL	1	2
1.0	5.0	10	100	10	1 mL	1	5
5.0	50.0	2.0 (slurry 16 g)	100	10	1 mL	1	10
50.0	100.0	1.0 (slurry 8 g)	100	10	1 mL	5	10
100.0	400.0	0.5 (slurry 4 g)	100	5	1 mL	2.5	10

(e) Cool the sample to room temperature. Quantitatively transfer the extracts into a 100 mL volumetric flask and make up to the mark with 0.15 M sodium phosphate buffer, mixing well.

(f) Transfer extracts into centrifuge tubes and centrifuge the samples at 4000 rpm for 15 min.

(g) Filter the samples using Whatman glass microfiber filter paper and collect the filtrate.

(h) Set up the SPE manifold. Attach the immunoaffinity column connected to a 10 mL reservoir. Drain off buffer just above the gel.

(i) Load the sample filtrate onto the column as per Table **2016.02A** and initialize the flow with the help of a vacuum pump.

(j) Let the solution pass through the column by gravity at a rate of one drop per second.

(k) Wash the column by passing 10 mL PBS through the column, followed by 10 mL water (initialize the flow with the help of vacuum at every step and leave it for gravity).

(l) Remove any residual liquid from the column by introducing gentle vacuum.

(m) Introduce a Reacti-Vial and elute the analyte under gravity with 2 mL methanol. Elute further with an additional 1 mL methanol. Backflush at least three times when eluting.

(n) Evaporate the eluent to dryness using a heating block set at 85 ± 5°C, under a gentle nitrogen blow down.

(o) Cool down to room temperature by keeping it outside for about 15 min

(p) Redissolve with 1 mL water and then cap the Reacti-Vials and vortex for 30 s. Filter by using a syringe filter in a clean glass insert for the HPLC analysis.

F. Standard Preparation

(a) *Stock Standard (100 µg/mL).*—Weigh 25 mg biotin reference material in a 250 mL amber volumetric flask. Add 150 mL water and sonicate at room temperature for 90 min with occasional shaking. Make up to volume with water.

(b) *Intermediate Standard (100 µg/100 mL).*—Dilute 1 mL stock standard to 100 mL with water.

(*1*) *Standard 1 (1.0 µg/100 mL).*—Dilute 100 µL intermediate standard to 10 mL with water.

(*2*) *Standard 2 (2.5 µg/100 mL).*—Dilute 250 µL intermediate standard to 10 mL with water.

(*3*) *Standard 3 (5.0 µg/100 mL)*.—Dilute 500 µL intermediate standard to 10 mL with water.

(*4*) *Standard 4 (7.5 µg/100 mL)*.—Dilute 750 µL intermediate standard to 10 mL with water.

(*5*) *Standard 5 (10 µg/100 mL)*.—Dilute 1 mL intermediate standard to 10 mL with water.

(*6*) *Standard 6 (20 µg/100 mL)*.—Dilute 2 mL intermediate standard to 10 mL with water.

G. Chromatographic Conditions

(a) *Mobile phase A*.—0.1% phosphoric acid.

(b) *Mobile phase B*.—100% acetonitrile.

(c) *Mobile phase C*.—80% acetonitrile.

(d) *Column*.—Kinetex Phenyl-Hexyl (Cat. No. 00F-4495-E0, Phenomenex, Torrance, CA), (150 × 4.6 mm × 2.6 µm × 100 Å).

(e) *Column temperature*.—25 ± 2°C.

(f) *Retention time*.—16 to 17 min.

(g) *Run time*.—27 min.

(h) *Detector*.—Photodiode Array Detector operating at 200 nm (spectrum scan 200–350 nm).

(i) *Injection volume*.—100 µL.

For Gradient program *see* Table **2016.02B**.

H. QC

(a) Check system suitability by injecting Standard 3 five times. The RSD, % should be ≤2%.

(b) Run the calibration standards at the beginning and end of the sequence (slope drift ≤2%).

(c) The six-point calibration should give a correlation coefficient ≥0.997.

(d) Test one in five samples in duplicate. The duplicates should be within the method repeatability.

(e) Inject one of the calibration standards after every five sample injections.

(f) Analyze a reference sample (e.g., National Institute of Standards and Technology Standard Reference Material 1849a) in duplicate.

(g) Identification of biotin peak is based on absolute retention time. Spectrum scan can be used for peak purity confirmation if required.

I. Calculation and Reporting

The chromatography software will automatically calculate the concentration of the sample in micrograms per 100 grams, provided the concentration of the standard in micrograms per 100 milligrams, sample weight (grams), and dilution are entered correctly.

Manual calculation can be performed by using the following equation:

$$\text{Biotin } (\mu g/100\ g) = \frac{(\text{Sample area} \times \text{volume in milliliters})}{(\text{Slope} \times \text{sample weight in grams})}$$

(The valid slope calculation is based on concentration on *x*-axis and area on *y*-axis.) Report results to three significant figures, using microgram-per-100-gram units or convert to other units as required.

Table 2016.02B. Gradient program

Time, min	Flow rate, mL/min	Mobile phase A, %	Mobile phase B, %	Mobile phase C, %
0.0	0.6	90	10	0
18.0	0.6	90	10	0
18.5	0.8	0	0	100
24.0	0.8	0	0	100
24.5	0.6	90	10	0
27.0	0.6	90	10	0

J. Repeatability

The difference between the results of duplicate portions of the same sample tested at the same sequence should not exceed 6% of the mean result.

K. Reproducibility

The difference between the results of duplicate determinations tested on different days should not exceed 12% of the mean result.

L. Uncertainty of Measurement

Uncertainty of the method was calculated as 7%, using appropriate statistical procedure (square root of the sum of squares of the errors expressed as a percentage).

M. LOQ

The LOQ was calculated based on the lowest working standard and dilution factor,

$$\text{LOQ} = (1 \times 100)/(20 \times 50) = 0.1 \text{ mg}/100 \text{ g } (1 \text{ ppb})$$

where 1 = 1 µg/100 mL lowest standard, 100 = volume (milliliters), 20 = 20 g sample, 50 represents the volume (milliliters) loaded on immunoaffinity column, and 1 = final volume (milliliters).

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OFFICIAL METHODS

Chloride in Milk, Milk Powder, Whey Powder, Infant Formula, and Adult Nutritionals by Potentiometric Titration Method: First Action 2016.03

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Introduction

The presented method is a combination of AOAC First Action methods **2015.07** (1) and **2015.08** (2). As both methods were reviewed by the Expert Review Panel as equivalent, the authors were asked to combine the methods into one method. This combined method maintains the performance requirements cited in each separate method single-laboratory validation and therefore meets the *Standard Method Performance Requirements* (3).

AOAC Official Method 2016.03
Chloride in Milk, Milk Powder, Whey Powder, Infant Formula, and Adult Nutritionals
Potentiometric Titration Method
First Action 2016

(Applicable to the determination of chloride in milk, milk powder, whey powder, infant formula, and adult nutritionals by potentiometry, with an analytical range of 0.35–1060 mg chloride/100 g reconstituted product or ready-to-feed (RTF) liquids).

Caution: Consult Material Safety Data Sheets for all substances that are required and considered hazardous. Follow all laboratory safety precautions and wear proper personal protective equipment.

A. Principle

Reconstitute powder samples by dissolving 25 g powder sample in 200 g warm water (40°C); RTF products are ready to use as they are. Precipitate proteins by adding precipitation solutions I and II, and then centrifuge. Acidify the supernatant with nitric acid solution. Titrate chloride ions against standardized silver nitrate solution (0.1 M), potentiometrically using a silver electrode to detect the end point.

B. Apparatus

Common laboratory equipment and, in particular, the following:

(a) *Analytical balance*.—Precision to 0.1 mg.

(b) *Centrifuge*.—Tabletop with rotor for 50 mL conical tubes, capable of operating at ≥12 000 × *g*.

(c) *Centrifuge tubes*.—50 mL, conical, polypropylene.

(d) *Pipets*.—1, 10, 20, 50, and 100 mL, Class A glass volumetric or automatic (Eppendorf or equivalent).

(e) *One-mark volumetric flasks*.—50, 100, 500, and 1000 mL, glass, Class A.

(f) *Graduated cylinders*.—25, 100, and 500 mL, glass.

(g) *Autosampler beaker*.—e.g., 120 mL, depending on the titrator used.

(h) *pH Meter/mV meter*.—With a scale covering ±700 mV, and a 20 or 25 mL buret (Mettler-Toledo, Columbus, OH), or equivalent.

(i) *Automatic titrator*.—Autosampler (Mettler-Toledo Rondo Tower) and motorized piston buret (Mettler-Toledo T50) with remote-control dispensing and filling (Mettler-Toledo LabX 3.1 software) or the Metrohm 862 Compact Titrosampler with 800 Dosino and 10 mL Exchange Unit (Riverview, FL), or equivalent. Alternatively, a semiautomated (e.g., Metrohm Titrado 905/907, with Metrohm *tiamo*™ software, or equivalent) or a manual titrator (using a buret with accuracy of 0.01 mL) may be used.

(j) *Combined ring silver electrode*.—e.g., Mettler DM 141 or DMi145-SC, Metrohm Ag Titrode No. 6.0430.100S, or equivalent. Alternatively, a silver electrode with reference electrode may be used.

(k) *Magnetic stirrer*.—Heidolph MR 3000 (Schwabach, Germany) or Metrohm 804Ti Stand with 802 Rod Stirrer, or equivalent.

(l) *Water bath*.—Capable of warming water to 40°C, or equivalent.

(m) *Ultrasonic cleaner*.—Model AS2060B (Tianjin Automatic Science Instrument Co. Ltd, Nanyang, China), or equivalent.

(n) *Disposable syringe*.—3 mL, with handspike and 0.45 µm disposable syringe filter.

C. Chemicals and Reagents

(a) *Water, purified*.—Greater than 18MΩ (EMD Millipore Corp., Billerica, MA), or equivalent.

(b) *Sodium chloride (NaCl)*.—Certified reference material for titrimetry, ≥99.5%, certified by the Federal Institute for Materials Research and Testing (Berlin, Germany) according to ISO 17025 (Cat. No. 71387; Sigma-Aldrich, St. Louis, MO), or equivalent.

(c) *Silver nitrate ($AgNO_3$)*.—Meets analytical reagent specification of the European Pharmacopoeia (Reag. Ph. Eur.), British Pharmacopoeia, and the United States Pharmacopeia (USP); assay 99.8–100.5% (Cat. No. 10220; Sigma-Aldrich); or equivalent.

(d) *Potassium ferrocyanide trihydrate [$K_4Fe(CN)_6$ $3H_2O$]*.—Grade puriss p.a., American Chemical Society (ACS), International Organization for Standardization (ISO), and Reag. Ph. Eur.; ≥99% (Cat. No. 31524; Sigma-Aldrich); or equivalent.

(e) *Zinc acetate dihydrate [$Zn(CH_3COO)_2$ $2H_2O$]*.—Grade ACS and puriss p.a., ≥99.0% (Cat. No. 96459; Sigma-Aldrich), or equivalent.

(f) *Nitric acid (HNO_3)*.—Minimum 65% puriss p.a. (Cat. No. 100452; Merck, Darmstadt, Germany), or equivalent.

(g) *Standardized $AgNO_3$ solution*.—0.1 mol/L (0.1 N) Titripur® grade Reag. Ph. Eur. and USP (Cat. No. 1.09081.1000 or EM3214-1; Merck, Darmstadt, Germany) or ready-to-use standardized titrant prepared according to GB/T 601-2002 (4), or equivalent.

(h) *Sodium chloride (NaCl) standardized solution, 0.1 M*.—Cat. No. 35616 (Alfa Aesar, Ward Hill, MA), or equivalent.

(i) *Glacial acetic acid, 100%*.—Anhydrous for analysis; EMSURE® grade ACS, ISO, and Reag. Ph. Eur. (Cat. No. 100063; Merck); or equivalent

(j) *Potassium nitrate (KNO_3)*.—For analysis, EMSURE® grade ISO and Reag. Ph. Eur. (Cat. No. 105063; Merck), or equivalent.

(k) *Acetone*.—For cleaning of the electrode (Cat. No. 010-4; Honeywell, Muskegon, MI), or equivalent.

(l) *Dimethylpolysiloxane*.—Defoaming agent (Cat. No. DMPS2C; Sigma-Aldrich), or equivalent.

D. Preparation of Solutions

(a) *Standardized $AgNO_3$ solution, 0.1 M*.—If ready-to-use $AgNO_3$ standard solution [**C**(**c**)] is not available, then weigh 16.9890 ± 0.0005 g $AgNO_3$ previously dried for 2 h at 120 ± 2°C. Dissolve in water and dilute to volume in a 1000 mL volumetric flask. Store in a brown reagent bottle.

Note 1: After preparation, check the titer by titration of 5.0 mL with exactly 0.1 M NaCl solution. For either commercial or in-house solution, verify the titer on a regular basis.

Note 2: The standardized $AgNO_3$ solution must be protected from light, and can be stored for up to 2 months.

(b) *Sodium chloride solution, 0.1 M*.—If ready-to-use NaCl standard solution is not available, weigh 5.8440 ± 0.0005 g NaCl [**C**(**b**)] previously dried for 2 h at 110 ± 2°C. Dissolve in water and dilute to volume in a 1000 mL volumetric flask.

Note: This solution is stable for up to 1 month.

(c) *Precipitating solution (Carrez) I*.—Weigh 106 g potassium ferrocyanide trihydrate [**C**(**d**)], dissolve in an appropriate amount of water, and transfer to a 1000 mL volumetric flask. Dilute to volume using water. Mix well.

(d) *Precipitating solution (Carrez) II*.—Weigh 220 g zinc acetate dihydrate [**C**(**e**)] and transfer to a 1000 mL volumetric flask. Dissolve with an appropriate amount of water and add 30 mL glacial acetic acid [**C**(**i**)]. Dilute to volume using water. Mix well.

(e) *Nitric acid solution*.—With care, add 100 mL concentrated nitric acid [**C**(**f**)] to 300 mL water. Mix well.

(f) *Wash solution*.—According to autosampler/titrator manufacturer's instructions [e.g., acetone or nitric acid solution (*see* **e**, above)], or other.

(g) *$AgNO_3$ solution, 0.025M (optional)*.—Into a 1000 mL volumetric flask, pipet 250 mL 0.1 M $AgNO_3$ solution [**C**(**g**) or **D**(**a**)]. Dilute to volume with water.

Note: Prepare freshly before use, and then check the titer by titration of 25 mL against 0.025 M NaCl solution.

(h) *NaCl solution, 0.025M (optional)*.—Into a 100 mL volumetric flask, pipet 25 mL 0.1 M NaCl solution [**D**(**b**)]. Dilute to volume with water.

Note: Prepare freshly before use.

(i) *KNO_3 solution, 1 M*.—Weigh 10.11 g potassium nitrate [**C**(**j**)] into a 100 mL volumetric flask. Add about 80 mL water and place the flask in an ultrasonic cleaner [**B**(**m**)] to dissolve with ultrasound and heating until dissolved thoroughly. Cool down

to room temperature and dilute to volume with water. Filter using a 0.45 µm membrane disposable syringe before use.

E. Sample Preparation

(a) *Milk product, infant formula, and adult/pediatric nutritional.*—Mix well to ensure that sample is homogeneous.

(b) *Powder samples.*—Reconstitute by dissolving 25 g powder sample in 200 g warm water (40°C).

F. Extraction

For high-protein samples requiring additional protein precipitation beyond that accomplished by addition of nitric acid solution, perform steps **F**(**a**–**c**), below. Otherwise, begin with step **F**(**d**).

(a) Weigh an appropriate aliquot of RTF or reconstituted powder (e.g., 25 g, accurate to 0.1 mg) into a 50 mL centrifuge tube. *Note*: For samples with a high chloride content, weigh a smaller test portion, e.g., 5 g reconstituted or RTF product.

(b) Transfer 2.5 mL precipitating solution I [**D**(**c**)] and 2.5 mL precipitating solution II [**D**(**d**)] into the tube. Dilute to 50 mL with water. Mix well. If foam impacts the constant volume, then one or two drops of defoaming agent [**C**(**l**)] should be added.

(c) Centrifuge at 12 000 × *g* for 5 min at 4°C, and then equilibrate to room temperature.

(d) Accurately transfer either 10 mL supernatant from steps **F**(**a**–**c**) or weigh an appropriate aliquot of RTF or reconstituted powder (e.g., 25 g, accurate to 0.1 mg). *Note*: For samples with a high chloride content, weigh a smaller test portion, e.g., 5 g reconstituted or RTF product.

Into a 120 mL sample beaker or autosampler cup, add 5 mL nitric acid solution [**D**(**e**)] and 50 mL water before titration.

Add a magnetic stirring rod (if the titrator does not have a built-in rod stirrer). Place the autosampler cup or beaker onto a magnetic stirrer and stir until dissolved or finely suspended.

(e) The pH of the test solution must be below 1.5. If in doubt, check pH by means of a pH meter and, if necessary, add a little more nitric acid solution [**D**(**e**)].

G. Instrument Operating Conditions

(a) *Check and maintenance of the combined silver electrode.*—Rinse electrode with deionized water and wipe before use. Renew the electrolyte with 1 M KNO_3 [**D**(**i**)] periodically per the manufacturer's recommendations.

If fat sticks to the electrodes during a series of analyses, then eliminate it by briefly immersing the electrode in acetone.

The silver electrode must be stored in 1 M KNO_3 [**D**(**i**)] after appropriate cleaning. *Note*: Instead of the combined silver electrode, separate silver and reference electrodes may also be used.

(b) *Titration.*—Connect the combined silver electrode to the titration apparatus according to the manufacturer's indications. Ensure that the titration vessels are correctly placed on the autosampler and that there are enough reagents: both nitric acid solution {if added automatically [**D**(**e**)]} and 0.1 M $AgNO_3$ [**C**(**g**) or **D**(**a**)]. If no autosampler is available, then place the sample solutions manually under the titration equipment.

Put the wash solution [**D**(**f**)] in the washing position if an auto sampler used. Ensure that the volume of wash solution is adequate.

Under continuous stirring and without touching the electrode, titrate the sample solution automatically with 0.1 M standardized silver nitrate solution [**C**(**g**) or **D**(**a**)] up to the end potential. The consumption of 0.1 M of silver nitrate solution [**C**(**g**) or **D**(**a**)] should be recorded automatically and can be read from the titrator software or documented in the titrator operating records. For manual titration, using a buret, add 0.1 M standardized silver nitrate solution [**C**(**g**) or **D**(**a**)] until the end potential has nearly been met. Continue to titrate slowly until the end point is met, as observed by the two small additions (about 0.05 mL) of silver nitrate solution. See Figures **201603A** and **2016.03B**.

(c) *Special case: determination of very low amounts of chloride.*—When determining low chloride concentrations such as found in desalted whey powder, for greater precision, it is preferable to use a standardized 0.025 mol/L $AgNO_3$ [**D**(**g**)] solution for the titration.

(d) *Blank test: determination of reagent background content of chloride.*—Perform a blank test using reagents, substituting water [**C**(**a**)] for the sample portion. The titrant consumption of the blank test obtained at the end point should be less than 0.05 mL when using the 0.1 M standardized silver nitrate, and less than 0.2 mL when using the 0.025 M standardized silver nitrate. Otherwise, check the reagents and water involved into the procedures and then perform the blank test again until the criterion is achieved.

H. System Suitability Test

Perform a system suitability test prior to use.

(a) Transfer 5 mL NaCl solution [**C**(**h**) or **D**(**b**)] into a 120 mL sample beaker. If 0.025 M $AgNO_3$ titrant is required, then use 1 mL NaCl solution.

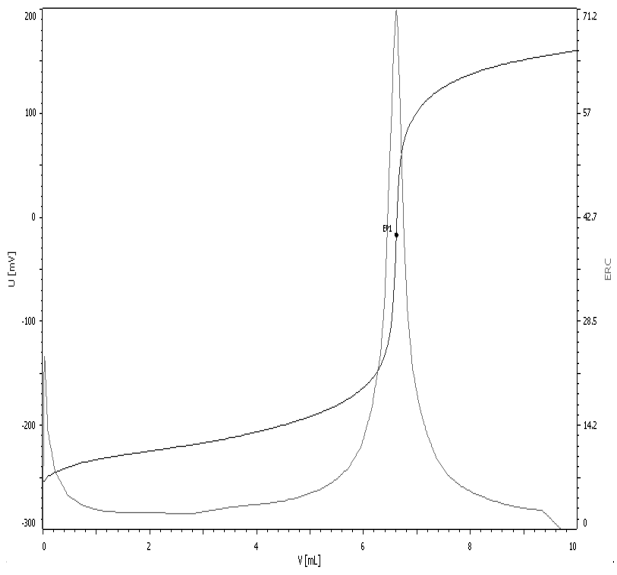
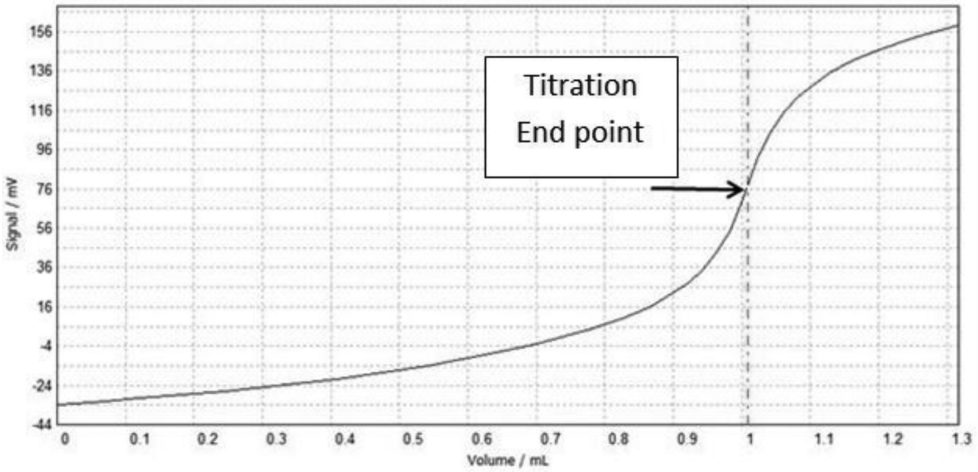


Figure 2016.03A. Automatic titration end point recognition using the dynamic titration mode on a Methohm Titrodo 905 titrator. U[mV], voltage of Ag electrode detected during titration; V[mV], volume of consumption of the standardized $AgNO_3$ titrant during titration; ERC, first derivative of the titration curve drawn by voltage of electrode versus volume of titrant consumption.



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 2016.03B. Example of titration curve from a Mettler autotitrator.

(b) Add 5 mL nitric acid solution [**D**(**e**)] and 50 mL water.

(c) Place the washing solution [**D**(**f**)] in the washing position of the auto sampler.

(d) Titrate with 0.1 M standardized silver nitrate solution [**C**(**g**) or **D**(**a**)] up to the end potential using an automatic, semiautomatic, or manual titrator.

(e) Repeat in quadruplicate.

(f) Calculate concentration of the silver nitrate solution according to section **I**(**a**). The difference between the calculated concentration and the certified value should be within 0.5%. If it is outside the acceptance value, check the experimental procedures and titration system. If the issue is not resolved, then use fresh standardized silver nitrate. If fresh standardized silver nitrate does not provide an acceptable result, replace the electrolyte of the electrode and check the operating condition of the dosing unit.

I. Calculations

(a) *Calculate silver nitrate concentration for system suitability verification, and report to four decimal places*.—If using in-house made standardized $AgNO_3$ solution [**D**(**a**)],

$$SNC(mol/L) = \frac{m_1}{5.844 \times V_1 \times 10}$$

where *SNC* is the silver nitrate concentration (mol/L), m_1 is the weight (mg) of sodium chloride in 5 mL or 1 mL standard solution [**D**(**b**)], V_1 is the volume (mL) of 0.1 M or 0.02 M $AgNO_3$ consumed at titration end point, 5.844 is the sodium chloride weight (µg) corresponding to 1 mL of 0.1 mol/L $AgNO_3$, and 10 is the mass conversion from titer to the concentration of titrant.

Or, if using purchased standard grade 0.10 M NaCl [**C**(**g**)],

$$SNC(mol/L) = \frac{0.1 \times V_3}{V_1}$$

where *SNC* is the silver nitrate concentration (mol/L), V_3 is the volume (mL) of purchased standard grade 0.10 M sodium chloride added, and V_1 is the volume (mL) of 0.1 M or 0.025 M $AgNO_3$ consumed at titration end point.

(b) *Calculate chloride content in the sample, and report to three significant digits*.—

$$CL(mg/100\,g) = \frac{35.45 \times c \times (V_2 - V_0) \times f \times 100}{m}$$

where: *CL* is the chloride content (mg/g); *m* is the sample weight (g); *c* is the certified concentration of silver nitrate titrant [0.1000 mol/L or standardized concentration; **I**(**a**)]; V_2 is the $AgNO_3$ volume (mL) consumed at titration end point; V_0 is the

$AgNO_3$ volume (mL) consumed at titration end point for Blank [**G**(**d**)]; *f* is the dilution factor for preparation of reconstituted powder, RTF, or concentrate {for samples requiring protein precipitation [**F**(**a**–**c**)], an additional factor (e.g., for a 25 g sample, *f* = 2) will be needed}; 35.45 is the chloride weight (µg) corresponding to 1 mL 1 mol/L $AgNO_3$; and 100 is the mass conversion to milligrams/100 g.

Under the repeatable analysis condition, the absolute difference between two independent test results should not exceed 2% of the arithmetic mean.

References

(1) *Official Methods of Analysis* (2016) 20th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **2015.07**

(2) *Official Methods of Analysis* (2016) 20th Ed., AOAC INTERNATIONAL, Rockville, MD, Method **2015.08**

(3) *Official Methods of Analysis* (2014) 19th Ed., AOAC INTERNATIONAL, Rockville, MD, AOAC SMPR 2014.015

(4) GB/T 601-2002 (2002) Chemical reagent – Preparation of standard volumetric solutions, http://www.chinesestandard.net/PDF-English-Translation/GBT601-2002.html

INFANT FORMULA AND ADULT NUTRITIONALS

Analysis of Vitamin D_2 and Vitamin D_3 in Fortified Milk Powders and Infant and Nutritional Formulas by Liquid Chromatography–Tandem Mass Spectrometry: Single-Laboratory Validation, First Action 2016.05

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A method for the determination of vitamin D_2 and vitamin D_3 in fortified milk powders and infant and adult nutritional formulas is described. Samples are saponified at high temperature and lipid-soluble components are extracted into isooctane. A portion of the isooctane layer is transferred and washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione is added to derivatize the vitamin D to form a high-molecular-mass, easily ionizable adduct. The vitamin D adduct is then re-extracted into a small volume of acetonitrile and analyzed by RPLC. Detection is by tandem MS, using multiple reaction monitoring. Stable isotope-labeled vitamin D_2 and vitamin D_3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatization and ionization efficiencies. A single-laboratory validation of the method using AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) kit samples was performed and compared with parameters defined according to the vitamin D *Standard Method Performance Requirements* (SMPR®). Linearity was demonstrated over the range specified in the SMPR, with the LOD being estimated at below that required. Method spike recovery (vitamin D_2, 97.0–99.2%; and vitamin D_3, 96.0–101.0%) and RSD_r (vitamin D_3, 1.5–5.2%) were evaluated and compared favorably with limits in the vitamin D SMPR. Acceptable bias for vitamin D_3 was demonstrated against both the certified value for National Institute of Standards and Technology 1849a Standard Reference material ($p_{(\alpha = 0.05)} = 0.25$) and AOAC INTERNATIONAL reference method 2002.05 ($p_{(\alpha = 0.05)} = 0.09$). The method was demonstrated to meet the requirements of the vitamin D SMPR as defined by SPIFAN, and was recently approved for Official First Action status by the AOAC Expert Review Panel on SPIFAN Nutrient Methods.

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This method was approved by the AOAC Expert Review Panel for SPIFAN Nutrient Methods as First Action.
The Expert Review Panel for SPIFAN Nutrient Methods 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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The major biological function of vitamin D is to maintain normal blood levels of calcium and phosphorus. Vitamin D aids in the absorption of calcium, helping to form and maintain strong bones, thereby preventing rickets in children (1). Vitamin D_3 (cholecalciferol) is generated in the skin of animals when a precursor molecule, 7-dehydrocholesterol, absorbs UV light energy. Thus, vitamin D is not a true vitamin because individuals with adequate exposure to sunlight do not require dietary supplementation. Infant formulas are typically fortified with vitamin D_3, and less commonly vitamin D_2, and are subject to strict regulatory control (2).

Accurate, precise, rapid, high-throughput analytical methods for vitamin D are needed for routine testing to ensure that products are manufactured within tight product specifications. Additionally, reference methods utilizing contemporary techniques are needed to guarantee product compliance with global regulations.

The described method was developed to provide an accurate, rapid, and robust technique for the routine compliance testing of vitamin D_3 in infant formulas and adult/pediatric nutritional formulas and was recently reported (3). To meet the requirements specified in the applicability statement of the vitamin D *Standard Method Performance Requirements* (SMPR®; 4), the scope of the analysis was extended to include vitamin D_2. As required by the AOAC Expert Review Panel (ERP) for Nutrient Methods Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) for endorsement as an Official First Action, method performance was evaluated in accordance with single-laboratory validation (SLV) procedures endorsed by the AOAC ERP (5).

In March 2016, this method and associated SLV data were assessed by the ERP and the method approved for Official First Action status. A recommendation by the ERP was added: The effect of temperature-induced interconversion of vitamin D and previtamin D, upon final results, should be investigated to provide evidence of the suitability of this method with respect to the applicability statement of the SMPR.

AOAC Official Method 2016.05
Analysis of Vitamin D_2 and Vitamin D_3 in Fortified Milk Powders, Infant Formulas, and Adult/Pediatric Nutritional Formulas
Liquid Chromatography–Tandem Mass Spectrometry
First Action 2016

[Applicable to the determination of vitamin D_2 and vitamin D_3 in fortified milk powders, infant formulas, and adult/pediatric nutritional formulas.]

Caution: Refer to the Material Safety Data Sheets for all chemicals prior to use. Use all appropriate personal protective equipment and follow good laboratory practices.

A. Principle

Samples are saponified at high temperature; then lipid-soluble components are extracted into isooctane. A portion of the isooctane layer is transferred and washed, and an aliquot of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) is added to derivatize vitamin D to form a high-molecular-mass, easily ionizable adduct. The vitamin D adduct is then re-extracted into a small volume of acetonitrile and analyzed by RPLC. Detection is by MS using multiple reaction monitoring (MRM). Stable isotope-labeled (SIL) *d6*-vitamin D_2 and *d6*-vitamin D_3 internal standards are used for quantitation to correct for losses in extraction and any variation in derivatization and ionization efficiencies.

B. Apparatus

(a) *Ultra-HPLC (UHPLC) system*.—Nexera (Shimadzu, Kyoto, Japan) or equivalent LC system consisting of a dual pump system, a sample injector unit, a degasser unit, and a column oven.

(b) *Triple-quadrupole mass spectrometer*.—Triple Quad 6500 (Sciex, Framingham, MA) or equivalent tandem MS (MS/MS) instrument.

(c) *Column*.—Kinetex C_{18} core-shell, 2.6 µm, 2.1 × 50 mm (Phenomenex, Torrance, CA) or equivalent.

(d) *UV spectrophotometer*.—Digital readout to three decimal places.

(e) *Centrifuge tubes*.—Polypropylene, 15 mL.

(f) *Boiling tubes*.—Glass, 60 mL.

(g) *Water baths*.—Cold 20°C, hot 70°C.

(h) *Disposable syringes*.—1 mL.

(i) *Syringe filters*.—PTFE, 0.2 µm, 13 mm.

(j) *Centrifuge*.—Suitable for 60 mL boiling tubes and 15 mL centrifuge tubes.

(k) *Pasteur pipet*.—Glass, ~140 mm.

(l) *Horizontal shaker*.

(m) *Eppendorf vials*.—2 mL.

(n) *Filter membranes*.—0.45 µm nylon.

(o) *Cryogenic vials*.—2 mL.

(p) *Schott bottles*.—1 L, 100 mL.

(q) *HPLC vials, septa, and caps*.

C. Reagents

(a) *Vitamin D_2 (ergocalciferol)*.—CAS No. 50-14-6, purity: ≥99%.

(b) *Vitamin D_3 (cholecalciferol)*.—CAS No. 67-97-0, purity: ≥99%.

(c) *d6-Vitamin D_2*.—(26,26,26,27,27,27-*d6* ergocalciferol), CAS No. 1311259-89-8, enrichment: ≥99%, purity: ≥99%.

(d) *d6-Vitamin D_3*.—(26,26,26,27,27,27-*d6* cholecalciferol), CAS No. 118584-54-6, enrichment: ≥99%, purity: ≥99%.

(e) *PTAD*.—Reagent grade (store in desiccator at 2–8°C).

(f) *Formic acid*.—LC–MS grade.

(g) *Potassium hydroxide*.—Reagent grade.

(h) *Magnesium chloride anhydrous*.—Reagent grade.

(i) *Pyrogallol*.—Reagent grade.

(j) *Ethanol*.—LC grade.

(k) *Methanol*.—LC–MS grade.

(l) *Isooctane (2,2,4-trimethylpentane)*.—LC grade.

(m) *Acetone*.—LC grade.

(n) *Acetonitrile*.—LC–MS grade.

(o) *Water*.—Reagent grade (≥18 MΩ).

D. Reagent Preparation

(a) *Acetone (dry)*.—To a 100 mL Schott bottle, add 50 mL acetone, then add ~10 g magnesium chloride to remove traces of moisture. Cap the bottle and seal with parafilm and wait for the magnesium chloride to settle before use (~24 h). Expiry: 1 month.

(b) *PTAD solution (10 mg/mL)*.—To a 5 mL volumetric flask, add 50 mg PTAD, then add 4 mL dry acetone, and dissolve; dilute to volume with acetone. Expiry: 1 day.

(c) *Potassium hydroxide solution (50%, w/v)*.—Dissolve 100 g potassium hydroxide in 200 mL water. Expiry: 1 month.

(d) *Ethanolic pyrogallol solution (1%, w/v)*.—Dissolve 5 g pyrogallol in 500 mL ethanol. Expiry: 1 day.

(e) *Mobile phase A (formic acid; 0.1%, v/v)*.—To 500 mL water, add 0.5 mL formic acid. Expiry: 1 week.

(f) *Mobile phase B (methanol; 100%, v/v)*.—500 mL methanol. Expiry: 1 month.

E. Standard Preparation

Because vitamin D is sensitive to light, perform all steps under UV-shielded lighting. If vitamin D_3 is exclusively required for analysis, then standards pertaining to vitamin D_2 need not be used and vice versa.

(a) *Stable isotope-labeled vitamin D_2 or vitamin D_3 stock standard ($SILD_2SS$ or $SILD_3SS$; ~10 µg/mL)*.—(*1*) Dispense the contents of a 1 mg vial of *d6*-vitamin D_2 or a 1 mg vial of *d6*-vitamin D_3 into separate 100 mL volumetric flasks.

(*2*) Dissolve in ~90 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol.

(*3*) Measure the absorbance of an aliquot of $SILD_2SS$ or $SILD_3SS$ at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Calculate and record the concentration.

(*4*) Immediately dispense aliquots of $SILD_2SS$ or $SILD_3SS$ (~1.3 mL) into cryogenic vials and freeze at ≤15°C.

(b) *Stable isotope-labeled internal standard (SILIS; ~1 µg/mL)*.—Make fresh daily.—(*1*) Prepare an adequate volume of SILIS for the daily sample numbers. For every 15 samples (or part thereof) in an analytical run, remove one

vial of $SILD_2SS$ and one vial of $SILD_3SS$ from the freezer and allow to warm to room temperature.

(*2*) Pipet 1.0 mL each of $SILD_2SS$ and $SILD_3SS$ into the same 10 mL volumetric flask (use a separate 10 mL volumetric flask for each set of 15 samples). Dilute to volume with acetonitrile and mix thoroughly.

(*3*) Pool all 10 mL volumetric flasks together and mix thoroughly.

(c) *Nonlabeled vitamin D_2 or vitamin D_3 stock standard (NLD_2SS or NLD_3SS; ~1 mg/mL).*—(*1*) Accurately weigh approximately 50 mg vitamin D_2 or vitamin D_3 into separate 50 mL volumetric flasks.

(*2*) Dissolve in ~40 mL ethanol. To promote dissolution, sonicate if necessary. Mix thoroughly; dilute to volume with ethanol. Store in a freezer at ≤15°C for a maximum of 3 months.

(d) *Nonlabeled vitamin D_2 or vitamin D_3 purity standard (NLD_2PS or NLD_3PS; ~10 µg/mL).*—Make fresh daily.—(*1*) Pipet 1.0 mL NLD_2SS or NLD_3SS into separate 100 mL volumetric flasks. Dilute to volume with ethanol.

(*2*) Measure the absorbance of an aliquot of each solution at 265 nm. The spectrophotometer should be zeroed against an ethanol blank solution. Record the absorbance and calculate the concentration.

(e) *Nonlabeled working standard (NLWS; ~1 µg/mL).*—Make fresh daily.—Pipet 1.0 mL NLD_2PS and 1.0 mL NLD_3PS into a single 10 mL volumetric flask. Dilute to volume with acetonitrile.

(f) *Calibration standards (CSs).*—Make fresh daily. *See* Table **2016.05A** for concentrations of the calibration standard solutions.—(*1*) *Calibration standard 1 (CS1).*—Pipet 10 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(*2*) *Calibration standard 2 (CS2).*—Pipet 50 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(*3*) *Calibration standard 3 (CS3).*—Pipet 250 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(*4*) *Calibration standard 4 (CS4).*—Pipet 500 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(*5*) *Calibration standard 5 (CS5).*—Pipet 1250 µL NLWS and 250 µL SILIS into a 25 mL volumetric flask.

(*6*) To each calibration standard, add 5 mL acetonitrile and 75 µL PTAD solution; shake to mix.

(*7*) Leave the calibration standards in the dark for 5 min.

(*8*) Add 6.25 mL water to each calibration standard and then dilute to volume with acetonitrile; shake to mix.

(*9*) Transfer ~1 mL of each calibration standard to an HPLC vial ready for analysis.

Table 2016.05A. Nominal concentrations of the calibration standards

	Concentration, ng/mL	
Calibration standard	Vitamin D	SIL *d6*-vitamin D
CS1	0.4	10
CS2	2.0	10
CS3	10	10
CS4	20	10
CS5	50	10

F. Sample Preparation

Because vitamin D is sensitive to light, perform all steps under UV-shielded lighting.

(a) *Powder sample preparation.*—Accurately weigh 1.8–2.2 g powder sample into a boiling tube. Record the weight.

(b) *Slurry sample preparation.*—(*1*) Accurately weigh 19.0–21.0 g powder into a disposable slurry container. Record the weight.

(*2*) Accurately weigh ~80 mL water into the container. Record the weight.

(*3*) Shake thoroughly until mixed. Place in the dark at room temperature for 15 min and shake to mix every 5 min.

(*4*) Accurately weigh 9.5–10.5 g slurry or reconstituted powder sample into a boiling tube. Record the weight.

(c) *Liquid sample preparation.*—Accurately weigh 10.0 mL liquid milk into a boiling tube. Record the weight.

G. Extraction and Derivatization

(a) To a powder, slurry, or liquid sample in a boiling tube, add 10 mL ethanolic pyrogallol solution, then add 0.5 mL SILIS, and then cap and vortex mix.

(b) Add 2 mL potassium hydroxide solution to the boiling tube; cap and vortex mix.

(c) Place the boiling tube in a water bath at 70°C for 1 h; vortex mix every 15 min.

(d) Place the boiling tube in a water bath at room temperature until cool.

(e) Add 10 mL isooctane to the boiling tube; cap the boiling tube tightly and place on a horizontal shaker for 10 min.

(f) Add 20 mL water to the boiling tube and invert the tube 10 times; place in a centrifuge at 250 × *g* for 15 min.

(g) Transfer a 5 mL aliquot of the upper isooctane layer into a 15 mL centrifuge tube using a Pasteur pipet, taking care not to transfer any of the lower layer.

(h) Add 5 mL water to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 2000 × *g* for 5 min.

(i) Transfer 4–5 mL upper isooctane layer to a new 15 mL disposable centrifuge tube using a disposable pipet, taking care not to transfer any of the lower layer.

(j) Add 75 µL PTAD solution to the centrifuge tube; cap and immediately vortex mix.

(k) Allow to stand in the dark for 5 min to allow the derivatization reaction to complete.

(l) Add 1 mL acetonitrile to the centrifuge tube; cap and vortex mix; then place in a centrifuge at 2000 × *g* for 5 min.

(m) Using a variable volume pipet, transfer 500 µL lower layer into an Eppendorf vial, taking care not to transfer any of the upper layer.

(n) Add 167 µL water to the Eppendorf vial; cap and vortex mix.

(o) Using a syringe filter, transfer an aliquot from the Eppendorf vial to an amber HPLC vial; then cap.

H. Chromatography

(a) Set up the UHPLC system with the configuration shown in Table **2016.05B**.

Table 2016.05B. Chromatographic instrument settings

Instrument parameter	Value
Mobile phase A	Formic acid, 0.1%
Mobile phase B	Methanol, 100%
Column	Kinetex C_{18}
Oven temperature	40°C
Chiller temperature	15°C
Injection volume	3 µL
Initial flow rate	0.6 mL/min

(b) Form gradients by high-pressure mixing of the two mobile phases, A and B, using the procedure in Table **2016.05C**.

I. Mass Spectrometry

(a) Set up the mass spectrometer with the instrument settings in Table **2016.05D**.

(b) The specific compound parameters to be used are listed in Tables **2016.05E** and **2016.05F**.

J. Calculations

(a) *Concentration of stable isotope-labeled vitamin D_2 in the stock standard, $SILD_2SS$.*—

$$SILD_2SS_{D2concn} = \frac{SILD_2SS_{abs(\lambda\ max)}}{E^{1\%}_{1\ cm}} \times 10000$$

where $SILD_2SS_{D2concn}$ is the concentration of *d6*-vitamin D_2 in the stock standard (µg/mL), $SILD_2SS_{abs(\lambda\ max)}$ is the UV absorbance of the stock standard at 265 nm (cm^{-1}), $E^{1\%}_{1\ cm}$ is the extinction coefficient for vitamin D_2 in ethanol (461 dL/g.cm), and 10 000 is the concentration conversion factor (g/dL to µg/mL).

(b) *Concentration of stable isotope-labeled vitamin D_3 in the stock standard, $SILD_3SS$.*—

$$SILD_3SS_{D3concn} = \frac{SILD_3SS_{abs(\lambda\ max)}}{E^{1\%}_{1\ cm}} \times 10000$$

where $SILD_3SS_{D3concn}$ is the concentration of *d6*-vitamin D_3 in the stock standard (µg/mL), $SILD_3SS_{abs(\lambda\ max)}$ is the UV absorbance of the stock standard at 265 nm (cm^{-1}), $E^{1\%}_{1cm}$ is the extinction coefficient for vitamin D_3 in ethanol (485 dL/g.cm), and 10 000 is the concentration conversion factor (g/dL to µg/mL).

Table 2016.05C. Mobile phase gradient

		Mobile phase composition	
Time, min	Flow rate, mL/min	A, %	B, %
0 start	0.6	25	75
3.3 pump	0.6	0	100
3.7 pump	1.0	0	100
4.8 pump	1.0	0	100
4.9 pump	0.6	25	75
5.5 stop	0.6	25	75

Table 2016.05D. Mass spectrometer instrument settings[a]

Instrument parameter	Value
Ionization mode	ESI^+
Curtain gas	30
Nebulizer gas GS1	40
Heater gas GS2	40
Collision gas	N_2
Source temperature	300°C
Ion spray voltage	5500 V

[a] These settings are suitable for the 6500 triple-quadrupole mass spectrometer (Sciex). Optimal settings on alternative instruments may differ.

(c) *Concentration of stable isotope-labeled vitamin D_2 in the internal standard, SILIS.*—

$$SILIS_{D2concn} = SILD_2SS_{D2concn} \times \frac{1.0}{10} \times 1000$$

where $SILIS_{D2concn}$ is the concentration of *d6*-vitamin D_2 in the internal standard (ng/mL), $SILD_2SS_{D2concn}$ is the concentration of *d6*-vitamin D_2 in the stock standard (µg/mL), and 1000 is the concentration conversion factor (µg/mL to ng/mL).

(d) *Concentration of stable isotope-labeled vitamin D_3 in the internal standard, SILIS.*—

$$SILIS_{D3concn} = SILD_3SS_{D3concn} \times \frac{1.0}{10} \times 1000$$

where $SILIS_{D3concn}$ is the concentration of *d6*-vitamin D_3 in the internal standard (ng/mL), $SILD_3SS_{D3concn}$ is the concentration of *d6*-vitamin D_3 in the stock standard (µg/mL), and 1000 is the concentration conversion factor (µg/mL to ng/mL).

(e) *Concentration of nonlabeled vitamin D_2 in purity standard NLD_2PS.*—

$$NLD_2PS_{D2concn} = \frac{NLD_2PS_{abs(\lambda\ max)}}{E^{1\%}_{1\ cm}} \times 10000$$

where NLD_2PSD_{2concn} is the concentration of vitamin D2 in the purity standard (µg/mL), and $NLD_2PS_{abs(\lambda\ max)}$ is the UV absorbance of the purity standard at 265 nm (cm^{-1}), $E^{1\%}_{1\ cm}$ is the extinction coefficient for vitamin D_2 in ethanol (461 dL/g.cm), and 10 000 is the concentration conversion factor (g/dL to µg/mL).

(f) *Concentration of nonlabeled vitamin D_3 in the purity standard, NLD_3PS.*—

$$NLD_3PS_{D3concn} = \frac{NLD_3PS_{abs(\lambda\ max)}}{E^{1\%}_{1\ cm}} \times 10000$$

where $NLD_3PS_{D3concn}$ is the concentration of vitamin D_3 in the purity standard (µg/mL), $NLD_3PS_{abs(\lambda\ max)}$ is the UV absorbance of the purity standard at 265 nm (cm^{-1}), $E^{1\%}_{1\ cm}$ is the extinction coefficient for vitamin D_3 in ethanol (485 dL/g.cm), and 10 000 is the concentration conversion factor (g/dL to µg/mL).

(g) *Concentration of nonlabeled vitamin D_2 in the working standard, NLWS.*—

$$NLWS_{D2concn} = NLD_2PS_{D2concn} \times \frac{1.0}{10} \times 1000$$

Table 2016.05E. Compound parameters (vitamin D_2 instrument method only)

Vitamin D_2 ion[a]	Precursor ion, *m/z*	Product ion, *m/z*	DP, V[b]	EP, V[c]	CE, V[d]	CXP, V[e]	Dwell time, ms
Analyte quantifier	572.2	298.0	81	10	23	22	120
Analyte qualifier	572.2	280.0	81	10	39	16	80
Internal standard quantifier	578.2	298.0	81	10	23	22	120
Internal standard qualifier	578.2	280.0	81	10	39	16	80

[a] The analyte is the vitamin D_2–PTAD adduct, and the internal standard ion is the *d6*-vitamin D_2–PTAD adduct.

[b] DP = Declustering potential.

[c] EP = Entrance potential.

[d] CE = Collision energy.

[e] CXP = Collision cell exit potential.

Table 2016.05F. Compound parameters (vitamin D_3 instrument method only)

Vitamin D_3 ion[a]	Precursor ion, *m/z*	Product ion, *m/z*	DP, V[b]	EP, V[c]	CE, V[d]	CXP, V[e]	Dwell time, ms
Analyte quantifier	560.2	298.0	151	10	21	18	120
Analyte qualifier	560.2	280.0	151	10	37	18	80
Internal standard quantifier	566.2	298.0	151	10	21	18	120
Internal standard qualifier	566.2	280.0	151	10	37	18	80

[a] The analyte is the vitamin D_3–PTAD adduct, and the internal standard ion is the *d6*-vitamin D_3–PTAD adduct.

[b] DP = Declustering potential.

[c] EP = Entrance potential.

[d] CE = Collision energy.

[e] CXP = Collision cell exit potential.

where $NLWS_{D2concn}$ is the concentration of vitamin D_2 in the working standard (ng/mL), $NLD_2PS_{D2concn}$ is the concentration of vitamin D_2 in the purity standard (µg/mL), and 1000 is the concentration conversion factor (µg/mL to ng/mL).

(h) *Concentration of nonlabeled vitamin D_3 in the working standard NLWS.*—

$$NLWS_{D3concn} = NLD_3PS_{D3concn} \times \frac{1.0}{10} \times 1000$$

where $NLWS_{D3concn}$ is the concentration of vitamin D_3 in working standard (ng/mL), $NLD_3PS_{D3concn}$ is the concentration of vitamin D_3 in purity standard (µg/mL), and 1000 is the concentration conversion factor (µg/mL to ng/mL).

(i) *Concentrations of vitamin D_2 and vitamin D_3 in calibration standards, CS1–CS5.*—

$$CS1_{Dconcn} = NLWS_{Dconcn} \times \frac{0.01}{25}$$

$$CS2_{Dconcn} = NLWS_{Dconcn} \times \frac{0.05}{25}$$

$$CS3_{Dconcn} = NLWS_{Dconcn} \times \frac{0.25}{25}$$

$$CS4_{Dconcn} = NLWS_{Dconcn} \times \frac{0.5}{25}$$

$$CS5_{Dconcn} = NLWS_{Dconcn} \times \frac{1.25}{25}$$

where CS1 through $CS5_{Dconcn}$ are the concentrations of vitamin D_2 or vitamin D_3 in the calibration standards (ng/mL), and $NLWS_{Dconcn}$ is the concentration of vitamin D_2 or vitamin D_3 in the working standard (ng/mL).

(j) *Concentrations of stable isotope-labeled d6-vitamin D_2 and d6-vitamin D_3 in the calibration standards, CS1–CS5.*—

$$CS1\text{–}5_{Dconcn} = SILIS_{Dconcn} \times \frac{0.25}{25}$$

where CS1 through $CS5_{Dconcn}$ are the concentrations of *d6*-vitamin D_2 or *d6*-vitamin D_3 in calibration standards (ng/mL), and $SILIS_{Dconcn}$ is the concentration of *d6*-vitamin D_2 or *d6*-vitamin D_3 in internal standard (ng/mL).

(k) *Mass of powder in slurried sample.*—

$$S_{mass} = \frac{D_{mass}}{(D_{mass} + W_{mass})} \times A_{mass}$$

where S_{mass} is the mass of the sample (g), D_{mass} is the mass of the dry powder used to make the slurry (g), W_{mass} is the mass of the water used to make the slurry (g), and A_{mass} is the mass of the aliquot of slurried sample used in the analysis (g).

(l) Determine the linear regression curves (vitamin D_2 and vitamin D_3) $y = m \cdot x + c$ (using the least-squares method) for the ratio of peak areas (nonlabeled vitamin D/stable isotope-labeled *d6*-vitamin D) versus the ratio of concentrations (nonlabeled vitamin D/stable isotope-labeled *d6*-vitamin D) for the five calibration standards, with the *y*-intercept forced through zero.

(m) The concentration (w/w) of vitamin D_2 or vitamin D_3 in the dry powders is calculated as

$$\text{Result D} = \frac{PA_{NLD}}{PA_{SILD}} \times \frac{SILIS_{Dconcn}}{L} \times \frac{SILIS_{alqt}}{S_{mass}} \times \frac{100}{1000}$$

where Result D is the vitamin D_2 or vitamin D_3 concentration in the sample (µg/h), PA_{NLD} is the peak area of vitamin D_2 or vitamin D_3 in the sample, PA_{SILD} is the peak area of *d6*-vitamin D_2 or *d6*-vitamin D_3 in the sample, $SILISD_{concn}$ is the concentration of *d6*-vitamin D_2 or *d6*-vitamin D_2 in the SILIS (ng/mL), L is the slope of the calibration curve, $SILIS_{alqt}$ is the volume of the SILIS aliquot spiked into the sample (0.5 mL), S_{mass} is the mass of the sample (g), 1000 is the mass conversion factor (ng/g to µg/g), and 100 is the mass conversion factor (µg/g to µg/hg).

(n) The concentration (w/v) of vitamin D_2 or vitamin D_3 in ready-to-feed (RTF) liquids is calculated as

$$\text{Result D} = \frac{PA_{NLD}}{PA_{SILD}} \times \frac{SILIS_{Dconcn}}{L} \times \frac{SILIS_{alqt}}{S_{vol}} \times \frac{100}{1000}$$

where Result D is the vitamin D_2 or vitamin D_3 concentration in the sample (µg/dL), PA_{NLD} is the peak area of vitamin D_2 or vitamin D_3 in the sample, PA_{SILD} is the peak area of *d6*-vitamin D_2 or *d6*-vitamin D_3 in the sample, $SILIS_{Dconcn}$ is the concentration of *d6*-vitamin D_2 or *d6*-vitamin D_2 in the SILIS (ng/mL), L is the slope of the calibration curve, $SILIS_{alqt}$ is the volume of the SILIS aliquot spiked into the sample (0.5 mL), S_{vol} is the volume of the sample (g), 1000 is the mass conversion factor (ng/g to µg/g), and 100 is the mass conversion factor (µg/g to µg/hg).

(o) The concentration of vitamin D_2 or vitamin D_3 as IU/hg in the sample is calculated as

$$\text{Result}\left(\text{IU/hg}\right) = \text{Result}\left(\text{µg/hg}\right) \times 40$$

where 40 is the dietary conversion factor (µg/hg to IU/hg).

K. Data Handling

Report results as µg/hg to one decimal place or as IU/hg to zero decimal places.

Results and Discussion

Method Optimization

The advantages of using the described derivatization strategy for the analysis of vitamin D are that many compounds (such as plant sterols) that are isobaric with vitamin D_2 and vitamin D_3 are excluded from detection because they lack the conjugated diene structure, and therefore do not form adducts. The derivatization of vitamin D with PTAD produces two epimers, 6S and 6R, because PTAD reacts with the *cis*-diene moiety from both the α-side and the β-side, with the ratio of 6S:6R being approximately 4:1 (6). The 6S/6R epimers coelute using the described chromatographic conditions, and the typical MRM chromatograms for a sample are shown in Figures 1 and 2.

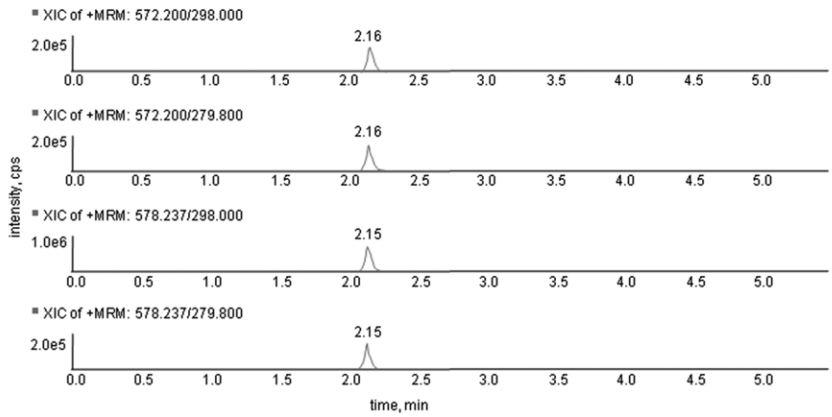

Figure 1. MRM chromatogram for vitamin D_2.

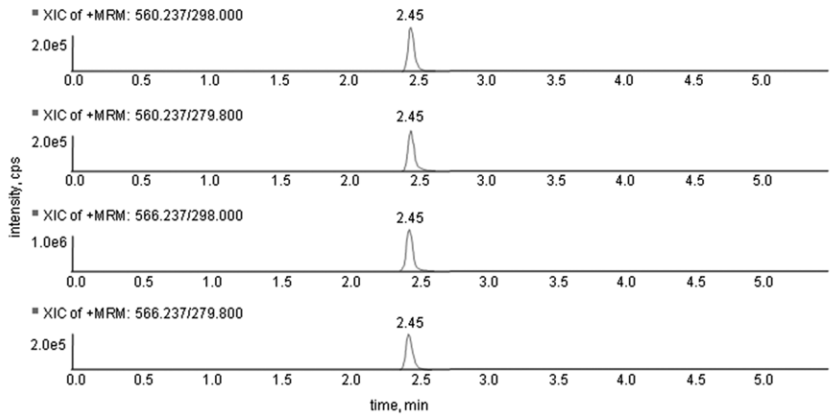

Figure 2. MRM chromatogram for vitamin D_3.

Product ion scans of the fragmentation of authentic vitamin D_3–PTAD $[M+H]^+$ and vitamin D_2–PTAD $[M+H]^+$ ions were performed (Figures 3 and 4). Product ions (298.0 and 280.0 *m/z*) were identified as being suitable quantifier and qualifier ion candidates for both vitamin D_2 and vitamin D_3. The method was optimized to enhance the signal of the transitions 572.2→298.0 and 572.2→280.0 for vitamin D_2 and the transitions 560.2→298.0 and 560.2→280.0 for vitamin D_3.

Single-Laboratory Validation

A wide range of infant formula and adult nutritional products that are available in the SPIFAN kit, plus an in-house vitamin D_3 QC milk powder sample, were used for the validation of this method (Table 1).

Linearity was evaluated by the analysis of six-level calibration standards on three different days. Visual inspection of the

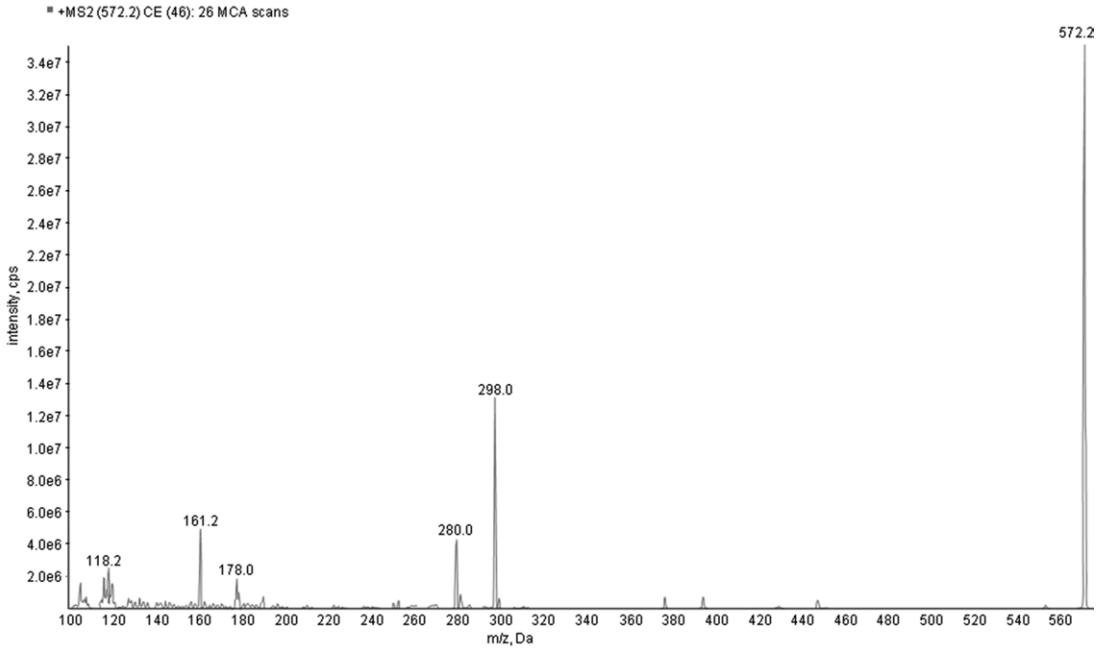

Figure 3. Product ion spectrum of vitamin D_2.

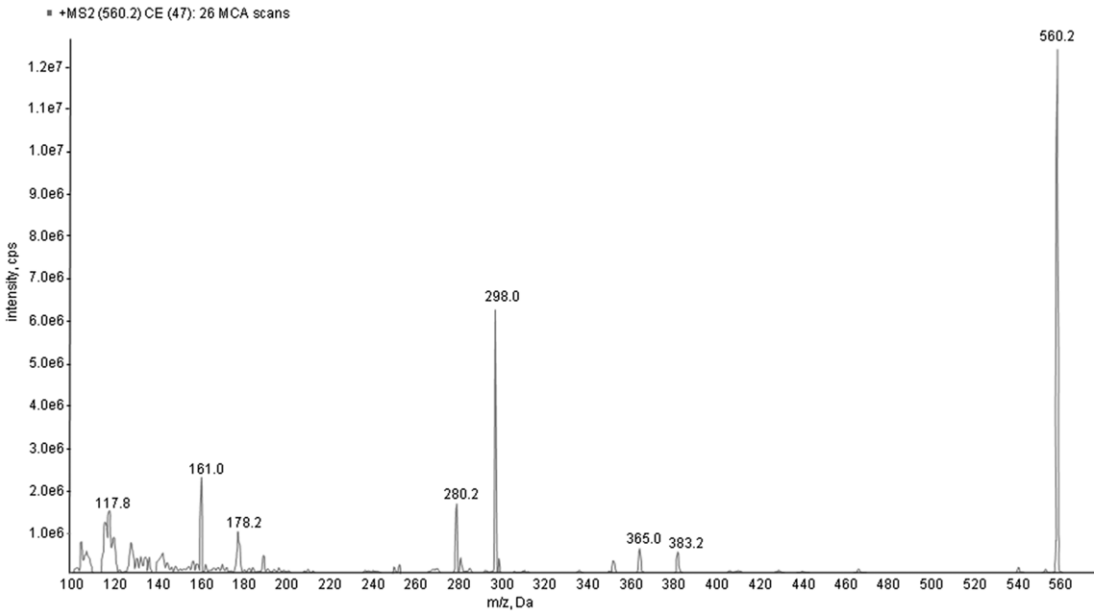

Figure 4. Product ion spectrum of vitamin D_3.

Table 1. Samples used during method validation

Sample description	Type	Code	Fortified
Child formula	Powder	00847RF00	No
Infant elemental	Powder	00796RF	No
Adult nutritional, high-protein	RTF	00821RF00	No
Adult nutritional, high-fat	RTF	00820RF00	No
Infant formula, milk-based	RTF	EV4H2Q	No
Infant formula, partially hydrolyzed, milk-based	Powder	410057652Z	Vitamin D_3
Infant formula, partially hydrolyzed, soy-based	Powder	410457651Z	Vitamin D_3
Toddler formula, milk-based	Powder	4052755861	Vitamin D_3
Infant formula, milk-based	Powder	4044755861	Vitamin D_3
Adult nutritional, low-fat	Powder	00859RF00	Vitamin D_3
Child formula	Powder	00866RF00	Vitamin D_3
Infant elemental	Powder	00795RF	Vitamin D_3
Infant formula FOS/GOS-based[a,b]	Powder	50350017W1	Vitamin D_3
Infant formula, milk-based	Powder	K16NTAV	Vitamin D_3
Infant formula, soy-based	Powder	E10NWZC	Vitamin D_3
Infant formula, milk-based	RTF[c]	EV4H2R	Vitamin D_3
Adult nutritional, high-protein	RTF	00730RF00	Vitamin D_3
Adult nutritional, high-fat	RTF	00729RF00	Vitamin D_3
NIST 1849a SRM[d]	Powder	CLC10-b	Vitamin D_3
In-house QC infant formula	Powder	—	Vitamin D_3

[a] FOS = Fructooligosaccharide.

[b] GOS = Galactooligosaccharide.

[c] RTF = Ready-to-feed.

[d] SRM = Standard Reference Material.

linear regression lines and residuals plots, back-calculation of standard concentrations (data not shown), and regression equations and correlation coefficients (Table 2) were used to demonstrate a linear relationship between instrument response and analyte concentration over the working range specified in the SMPR. The linear ranges for vitamin D_2 and vitamin D_3 extended beyond both the lower limit and the upper limit of the range specified in the vitamin D SMPR.

Precision was assessed for all of the fortified samples by testing duplicate samples on six separate days by two different analysts on a single instrument, with fresh calibration standards and reagents being made each day (Table 3). The repeatability of the method for the SPIFAN kit samples ranged between 1.5 and 5.2%, which complied with the ≤11.0% limit set in the SMPR. The HorRat values were within acceptability criteria for repeatability of 0.3–1.3 (7). Intermediate precision ranged between 3.1 and 7.9%, with a mean value of 5.5%, less than the 15% limit for reproducibility defined in the SMPR.

Table 2. Linearity and range for vitamin D_2 and vitamin D_3

Analyte	Linear regression	Correlation coefficient	Range, ng/mL	Range as RTF, µg/hg[a]	SMPR limits, µg/hg
Vitamin D_2	$y = 0.87x + 0.015$	1.0000	0.3–59.1	0.04–7.3	
Vitamin D_3	$y = 0.87x + 0.015$	0.9999	0.5–92.8	0.06–11.3	0.12–5.1

[a] RTF = Ready-to-Feed at a concentration of 25 g dissolved in 200 mL.

Table 3. Repeatability and intermediate precision of the method for vitamin D

Sample	Repeatability RSD, % (HorRat)	Intermediate precision RSD, %
Infant formula, partially hydrolyzed, milk-based	4.4 (0.2)	7.4
Infant formula, partially hydrolyzed, soy-based	1.8 (0.1)	5.0
Toddler formula, milk-based	2.2 (0.1)	4.4
Infant formula, milk-based	2.1 (0.1)	4.4
Adult nutritional, low-fat	3.7 (0.1)	6.3
Child formula	3.3 (0.1)	5.8
Infant elemental	3.5 (0.1)	3.1
Infant formula, FOS/GOS-based[a,b]	1.5 (0.1)	4.7
Infant formula, milk-based	3.3 (0.1)	6.4
Infant formula, soy-based	2.6 (0.1)	3.6
Infant formula, milk-based	2.3 (0.1)	7.8
Adult nutritional, high-protein	1.6 (0.1)	5.2
Adult nutritional, high-fat	4.9 (0.2)	7.9
NIST 1849a SRM[c]	2.8 (0.1)	5.4
In-house QC infant formula	5.2 (0.2)	5.4

[a] FOS = Fructooligosaccharide.

[b] GOS = Galactooligosaccharide.

[c] SRM = Standard Reference Material.

The LOD and LOQ were initially estimated by evaluating multiple whole-milk powder samples spiked at a range of concentrations and by determining the spike concentration that gave an S/N of approximately 10. This was determined to be a concentration of 2 ng of vitamin D spiked into a 2 g sample. The LOD and the LOQ were then determined from 10 independent analyses. The LOD and the LOQ for vitamin D_2 were determined to be 0.12 and 0.15 µg/hg, which were equivalent to 0.013 and 0.016 µg/hg as RTF, specified in the SMPR. The LOD and the LOQ for vitamin D_3 were determined to be 0.16 and 0.25 µg/hg, equivalent to 0.018 and 0.028 µg/hg as RTF. The LOD and the LOQ for both vitamin D_2 and vitamin D_3 were lower than those defined in the vitamin D SMPR.

Recovery was evaluated using unfortified samples within the SPIFAN kit. Each matrix was spiked at two levels: 50% (5 µg/hg ≈ 0.6 µg/hg RTF) and 100% (10 µg/hg ≈ 1.1 µg/hg RTF) of typical infant formula concentrations. Spike samples were analyzed on three separate days. The mean recoveries measured were between 97.0 and 99.2% for vitamin D_2 and between 96.0 and 101.0% for vitamin D_3 (Table 4), within the limits set in the SMPR of 90–110%.

Table 4. Recoveries for vitamin D_2 and vitamin D_3

	Recovery, % (RSD, %)	
Sample	Vitamin D_2	Vitamin D_3
Child formula powder	99.2 (3.7)	100.5 (2.2)
Infant elemental powder	97.6 (1.5)	97.0 (1.4)
Adult nutritional RTF, high-protein[a]	98.5 (1.1)	97.7 (0.9)
Adult nutritional RTF, high-fat	98.3 (2.3)	101.0 (2.7)
Infant formula RTF, milk-based	97.0 (3.1)	96.0 (2.0)

[a] RTF = Ready-to-feed.

Table 5. Results for the bias experiment against NIST 1849a SRM[a]

Parameter	Value
Certified value, µg/hg	11.1
Uncertainty, µg/hg	1.7
Certified range, µg/hg	9.4–12.8
Coverage factor, *k*	2
Degrees of freedom, DF_{CRV}	60
Mean, $\bar{x}$	10.1
SD	0.53
Number of replicates, *n*	13
95% Confidence interval, µg/hg	9.8–10.4
T_{stat}	1.165
Degrees of freedom	63.92
$p_{(\alpha = 0.05)}$	0.25

[a] SRM = Standard Reference Material.

Bias was evaluated by replicate analyses of the National Institute of Standards and Technology (NIST) 1849a Standard Reference Material (SRM). Differences between the measured value and the certified value were determined with the mean and SD of the differences, and the test statistic was calculated. A $p_{(\alpha = 0.05)}$ of 0.25 indicates that there was no bias between the measured results and the certified value (Table 5). As part of initial method validation, the LC-MS/MS was evaluated for bias against an HPLC–UV method based on AOAC **2002.05** (8, 9). A $p_{(\alpha = 0.05)}$ of 0.09 indicates that there was no bias between the methods (Table 6). Bias against a certified reference material or a reference method is not a defined parameter within the SMPR.

Vitamin D–Previtamin D Interconversion

Although the described method specifically detects vitamin D and not the previtamin D isomer, the method quantifies an aggregate result for both previtamin D and vitamin D. This satisfies the requirement of the applicability statement of the SMPR, which specifies total vitamin D_2 or vitamin D_3, including their previtamin isomers. It was assumed in this analysis, as with all analytical methods for vitamin D that use calciferol internal standards, that the previtamin D:vitamin D ratio was equivalent for the sample analyte and the internal standard. For deuterated internal standards, the labeled site must be remote from the triene center because of the difference in interconversion behavior between the analyte and the internal standard (10). To confirm this assumption, the effect of temperature on the final results was evaluated. Experiments were performed with saponification assessed in three different ways: (*1*) at 70°C for 1 h, according to the described method protocol; (*2*) at 20°C for 7.5 h; and (*3*) at 70°C for 7.5 h. A 7.5 h saponification was chosen because this is the time needed, as previously reported, for a pure solution of vitamin D to reach equilibrium with previtamin D at 70°C (11). Samples 1–6 and 13–18, which were saponified at 70°C, showed significantly lower absolute peak areas for the vitamin D–PTAD quantifier ion than samples 7–12, which were saponified at 20°C. This was as expected because a higher proportion of vitamin D is converted to previtamin D at the elevated temperature. This effect was seen for both the analyte vitamin D in the sample and the SIL *d6*-vitamin D internal standard, illustrating the appropriateness of the internal standard to account for any temperature-induced interconversion between previtamin D and vitamin D (Figure 5). The final results obtained showed that, within sample error, there

Table 6. Results for the bias experiment against AOAC 2002.05

Parameter	Reference method	LC-MS/MS method
Mean, µg/hg	10.5	10.8
SD, µg/hg	3.18	3.66
Number of replicates, *n*	40	40
95% Confidence interval, µg/hg	10.0–11.0	10.2–11.4
Mean of paired differences	–0.3	
SD of paired differences	1.27	
T_{stat}	1.73	
Degrees of freedom	38	
$p_{(\alpha = 0.05)}$	0.09	

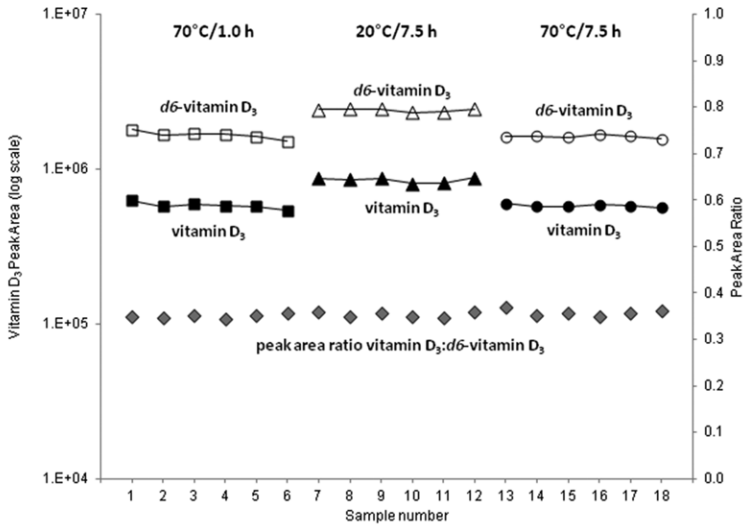

Figure 5. Effect of saponification time/temperature on vitamin D and *d6*-vitamin D.

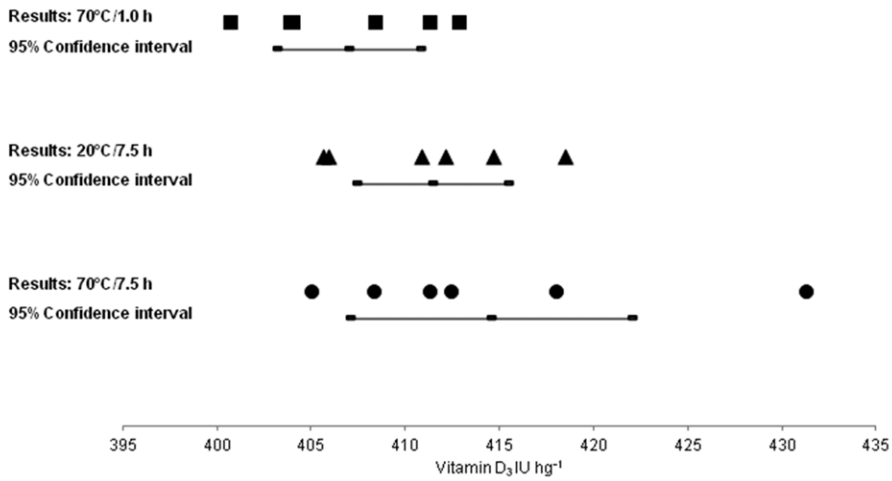

Figure 6. Effect of saponification time/temperature on the measured results for vitamin D.

was no difference between the three experiments, which was consistent with the premise that the described method measures an aggregate result for both previtamin D and vitamin D forms (Figure 6).

The separate measurement of previtamin D was investigated as part of an independent initial method proof of concept and in which a number of practical reasons for not quantifying previtamin D separately were discussed (12). Its inclusion as part of the analysis would add complexity, with no material improvement to the estimation of vitamin D because (*1*) the relative ionization and fragmentation efficiencies of vitamin D–PTAD and previtamin D–PTAD are not known; (*2*) the previtamin D–PTAD peak has a different retention time from the vitamin D–PTAD peak and may be subject to different ion suppression, thereby making accurate quantitation of this form difficult; and (*3*) a pure standard for previtamin D is not available (12).

It has been demonstrated that separate detection and measurement of previtamin D in this method was not necessary and that the results obtained would be consistent with the requirements of the SMPR.

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Fructans in Infant, Adult, and Pediatric Nutritional Formulas: Single-Laboratory Validation, First Action 2016.06

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A method for fructan analysis designed to comply with AOAC *Standard Method Performance Requirements* (SMPR®) 2014.002 is described. It is closely related to existing methods for fructan analysis, including AOAC 997.08 and 999.03, as well as a method previously published by Cuany et al. This new method achieves LOQ of 0.03% fructan on a ready-to-feed (RTF) basis with mean recoveries ranging from 93 to 108% in the presence of up to 9% sucrose (even at the 0.03% level of fructan). Repeatability ranged from 1.09 to 3.67%. Intermediate precision ranged from 2.46 to 6.79%. Sample preparation for quantitative analysis is simplified compared to some of the existing methodologies. The method incorporates a qualitative profile analysis to determine fructan size category. This allows assignment of appropriate correction factors without independent knowledge of fructan type.

As defined in AOAC SMPR 2014.002 (1), fructan is a general term that encompasses fructooligosaccharides, oligofructose, and inulin. These are all referred to as inulin-type fructans, despite the fact that ingredient sources relevant to this category may not necessarily be derived from inulin. These carbohydrates act as dietary fiber with prebiotic benefits and range in size from a degree of polymerization (DP) of 2 to 100. Fructans of this type are represented by two general structural forms (Figure 1). Fructooligosaccharides and intact inulin materials are comprised almost exclusively of GF_n type molecules (i.e., an oligosaccharide composed of a chain of n fructose molecules with a terminal glucose molecule). Oligofructose and materials that are a mix of intact inulin and oligofructose contain both GF_n and F_m type molecules (F_m meaning an oligosaccharide composed of a chain of m fructose molecules only). GF_n structures are non-reducing while F_m structures are, and the reducing nature of the latter has significant ramifications for methodology capable of determining both types.

Because relevant ingredients are all mixtures of varying complexity, methodology based on direct determination of all the fructan forms that are present is of limited utility, especially in complex nutritional formulations. High-temperature GC methods (2, 3) and HPAEC/PAD profiling methods (4, 5) have been reported, but the lack of suitable individual reference standards limits the usefulness of methods attempting direct quantitative determination of the entire fructan profile, as does the potential presence of a complex non-fructan carbohydrate system. In addition, the direct profiling methods are generally limited to species of DP <5–8 (for the methods noted above). In one approach, profiling of test samples is used to tentatively identify the specific fructan ingredient. Subsequent quantitative analysis is then based on determination of one or a few "marker" components using a calibration curve constructed from analysis of actual commodity samples. While this type of strategy can produce accurate results under ideal circumstances, there are significant practical limitations, one of which is the growing diversity of ingredients (and suppliers). It also fails to account for the fact that even lot-to-lot differences in ingredient fructan profile is a potential source of uncertainty as well as the possibility that the final fructan profile in a food product may differ from that in the original ingredient due to changes incurred during processing. As a result of the complexities associated with direct determination, methods generally emphasize a strategy based on determination of the monosaccharides released from fructans by enzymatic hydrolysis and subsequent calculation of fructan content using appropriate correction factors.

Methods based on post hydrolysis analysis of monosaccharides may rely on determination of both glucose and fructose or fructose only. Both AOAC **997.08** (6) and AOAC **999.03** (7) determine glucose *and* fructose. In such methods the only correction factor required is for water added during hydrolysis:

$$C_{fructan} = k_W (C_{G,f} + C_{F,f}) \quad (1)$$

where $C_{fructan}$ = fructan concentration; $C_{G,f}$ = concentration of glucose from fructan; $C_{F,f}$ = concentration of fructose from fructan.

$$k_w = \text{correction factor for water} = 0.9 + \frac{0.1}{DP_{avg}}$$

where DP_{avg} = average DP of fructan.

The presence of reducing sugars negatively impacts AOAC method **999.03** (7). Because the reducing end of the molecule of F_m forms is converted to a sugar alcohol, it will not react with the PAHBAH reagent (*p*-hydroxybenzoic acid hydrazide) used

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This method was approved by the AOAC Expert Review Panel for SPIFAN Nutrient Methods as First Action.
The Expert Review Panel for SPIFAN Nutrient Methods 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: philip.haselberger@abbott.com
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$$GF_n \text{ Type: } (\beta - DFruf - (2 \rightarrow 1))_n - \beta - D - Fruf - (2 \rightarrow 1) - \alpha - D - Glcp$$

$$F_m \text{ Type: } (\beta - DFruf - (2 \rightarrow 1))_m - (2 \rightarrow 1)\text{-D- } Fruf$$

Fru*f* = fructofuranose
Glc*p* = glucopyranose

Figure 1. General formulae for the two major inulin-type fructans relevant to SMPR 2014.002.

in the post hydrolysis colorimetric determination of fructan-derived monosaccharides. As a result, the total fructan content will be underestimated. AOAC **997.08** relies on a correction for the monosaccharides released by other carbohydrates, including sucrose (6). This results in compromised precision because of the error propagation in the background corrections which generally limits the usefulness of method **997.08**, or similar methods, to samples with a sucrose:fructan ratio of 3:1 or 4:1 (or less; 8, 9).

Methods based on determination of fructan from only fructose require two correction factors:

$$C_{fructan} = k_W k_G (C_{F,f}) \quad (2)$$

$$k_G = \text{correction for glucose content} = \frac{q+1}{q}$$

where q = average fructose to glucose ratio of fructans.

For the special case of GF_n fructans:

$$k_G = \left(\frac{DP_{avg}}{DP_{avg^{-1}}}\right) \quad (3)$$

which makes the overall correction ($k_W k_G$), in terms of DP_{avg}:

$$k_W k_G = \left(\frac{0.9DP_{avg} + 0.1}{DP_{avg^{-1}}}\right) \quad (4)$$

The method of Cuany et al., recognizes that, when sodium borohydride treatment is used to eliminate free reducing sugars, Equation 4 applies to fructans of both GF_n and F_m type because, if fructan is calculated on the basis of fructose only, the terminal glycosyl residue is lost for all forms, just as it would be for only GF_n species (10). The shortcoming of this method, as with all methods to date, is the need to either have independent knowledge of the fructan ingredient (which would allow selection of a specific correction factor) or application of a common factor regardless of fructan type. The method presented here is based on the chemistry used in the Cuany method (10), but with a few distinct differences, the most important being the addition of a procedure for selecting the appropriate correction factor to be applied for calculating the total fructan content and significant procedural simplification by eliminating the need for SPE cleanup.

Table 2016.06A. Total fructan single-laboratory validation data: precision

Sample type	No. of replicates	Mean, g/100g, RTF[a]	SD_r	RSD_r, %	SD_{IP}[b]	RSD_{IP}, %[c]
Materials from SPIFAN sample kit						
Child formula powder, placebo	12[d]	0.270	0.0055	2.03	0.01547	5.73
Toddler formula powder, milk based	12[d]	0.233	0.0080	3.42	0.00806	3.46
Infant formula powder, milk based	12[d]	0.283	0.0059	2.09	0.00696	2.46
Child formula powder	12[d]	0.277	0.0072	2.61	0.01238	4.47
Infant formula powder with FOS/GOS[e]	12[d]	0.036	0.0008	2.14	0.00117	3.29
Adult nutritional RTF high fat	12[d]	0.500	0.0184	3.67	0.03395	6.79
Abbott Nutrition in-house materials						
Infant formula powder, soy based	40[f]	0.153	0.0025	1.64	0.00442	2.89
Adult nutritional powder	40[f]	0.434	0.0060	1.38	0.01167	2.69
Pediatric powder, milk based	40[f]	0.230	0.0052	2.24	0.00892	3.88
Control powder, milk based	60[g]	0.370	0.0040	1.09	0.00951	2.57

[a] RTF = Ready-to-feed.
[b] SD_{IP} = Standard deviation (intermediate precision).
[c] RSD_{IP} = Relative standard deviation (intermediate precision).
[d] Duplicates on each of 6 days, one laboratory.
[e] FOS/GOS = Fructooligosaccharides/galactooligosaccharides.
[f] Duplicates on each of 10 days, in each of two laboratories.
[g] Duplicates on each of 10 days, in each of three laboratories.

Table 2016.06B. Total fructan single-laboratory validation data: Recovery from overspiked SPIFAN samples

Sample type	No. of replicates[a]	Spiking material[b]	Native fructan level, g/100 g[c]	50% Overspike		100% Overspike	
				Avg. % rec.	RSD, %	Avg. % rec.	RSD, %
Child formula powder	6	scFOS	0.277	97.2	6.28	100	3.74
Toddler formula powder, milk based	6	Oligofructose	0.233	108	4.38	105	15.0
Infant formula powder, milk based	6	Oligofructose	0.283	101	8.53	98.7	4.82
Child formula powder	6	scFOS	0.277	102	4.78	96.0	5.75
Infant formula powder FOS and GOS	6	Oligofructose	0.036	97.4	11.1	97.6	7.91
Adult nutritional RTF high fat	6	scFOS	0.500	92.9	4.91	94.2	12.6

[a] Duplicates on each of 3 days.
[b] From SPIFAN kits. scFOS = Ingredion Nutraflora FOS powder. Oligofructose = Beneo Orafti P95 powder.
[c] Mean from previous precision determination, duplicates on each of 6 days.

AOAC Official Method 2016.06
Fructans in Infant, Pediatric, and Adult Nutritional Formulas
HPAEC/PAD
First Action 2016

[Applicable to the determination of the total fructan content in infant, pediatric, and adult nutritional products (as well as commodities) as defined in SMPR 2014.02 (1)].

See Tables **2016.06A–C** for matrixes for which SLV data has been generated, supporting acceptance of the method.

A. Principle

A two-part analysis is performed. Part I is a qualitative classification of the fructan present in the sample. This classification is based on rules related to presence/absence of GF_3 (nystose) and/or GF_4 (fructofuranosylnystose), specifically, and higher oligomers, generally. It groups the fructans into one of three DP ranges and allows assignment of an appropriate commodity factor for calculating the total fructan concentration based on the amount of fructose released by enzymatic hydrolysis of the fructans.

Part II is the quantitative determination of the fructan-derived fructose, from which the total fructan in calculated. The sample to be analyzed is weighed and diluted with laboratory water, as appropriate. Part I of the analysis is then performed. Part II can be run in parallel, whereby an aliquot of the diluted sample is treated with sucrase to hydrolyze any sucrose present. Glucose and fructose released by the sucrase treatment, as well as inherent glucose and fructose, are then reduced to sugar alcohols by the addition of sodium borohydride. Excess borohydride is neutralized by the addition of acetic acid. Lastly, an aliquot of internal standard (IS; glucoheptose) and an aliquot of fructanase are added to the sample solution. After the fructanase hydrolysis is completed, the samples are analyzed for fructose on HPAEC-PAD instrumentation. The total fructan content is calculated from the fructose, adjusted by the commodity factor, *CF,* determined by Part I (if not known).

Part I - Fructan Classification for Determination of Commodity Factor

B. Apparatus and Materials

(a) *LC system.*—One biocompatible gradient pump; biocompatible refrigerated autosampler capable of injecting 4 µL; electrochemical detector with gold electrode capable of pulsed amperometric detection. For example, Dionex ICS-3000 or 5000 (Dionex Corp., Sunnyvale, CA) consisting of an SP, DP, WPS-3000 (TB; P or S type), and an electrochemical cell with a conventional gold working electrode and combination pH Ag/AgCl reference electrode or equivalent.

(b) *Analytical column set.*—Analytical column, Thermo CarboPac PA1, 250 × 4 mm PA1 guard column.

(c) *Analytical balance.*—Readable to 0.01 mg.

(d) *Syringe filter.*—0.45 µm nylon.

(e) *Volumetric flasks.*—Glass, Class A, assorted sizes.

(f) *Eluent filtration apparatus with disposable membrane filter.*—0.2 µm polyethersulfone (PES).

(g) *Helium sparge.*—Tubing and frit assembly.

Table 2016.06C. Total fructan single-laboratory validation data: Recovery from spiked SPIFAN placebo samples

Sample type	No. of replicates[a]	Spiking material[b]	Spike level, g/100 g RTF	Avg. % rec.	RSD, %
Infant formula powder partially hydrolyzed soy based	6	Inulin	0.120	97.4	4.25
Infant formula powder partially hydrolyzed soy based	6	Inulin	0.353	102	4.25
Adult nutritional powder low fat	6	Oligofructose/inulin	0.133	98.1	4.65
Adult nutritional powder low fat	6	Oligofructose/inulin	0.378	95.9	3.82

[a] Duplicates on each of 3 days.
[b] Inulin = Beneo Orafti HP powder (SPIFAN kit). Oligofructose/inulin = Beneo Orafti Synergy 1.

Table 2016.06D. Sample size guidelines

Sample type	Fructan level (as-is), %	SW_1, g	Volumetric flask size, mL[a]
Powder product	0.27–1.0	10 g ± 10%	50
	1.1–10	7 g ± 10%	200
	11–45	4 g ± 10%	500
RTF product[b]	0.03–0.20	10 g ± 10%	50
	0.21–1.0	7 g ± 10%	200
	1.1–5.0	4 g ± 10%	500
Commodity	45–100	0.4 g ± 10%	1000

[a] For the sake of procedural simplification, the density of this solution is treated as 1 g/mL, so that the weight of the diluted solution in grams can be assumed equal to the flask volume (in mL). This incurs a <0.3% error, which is inconsequential for a result reported to 3 significant digits.

[b] For concentrated liquid (CL) products dilute 10 mL of product with 10 mL of laboratory water and then treat the diluted material per RTF guidelines.

C. Reagents

(a) *Laboratory water.*—ASTM Type 1.

(b) *NaOH solution.*—50%, w/w (Fisher Part No. SS254-500 or equivalent). If an alternate vendor is selected, it is imperative that the carbonate level be equivalent to or less than Fisher (≤0.10%).

(c) *Sodium acetate trihydrate.*—Reagent grade (Sigma Part No. 71188 or equivalent).

(d) *Helium gas.*—Ultra-high purity (UHP).

(e) *Fructan commodities.*—For use as retention time (RT) standards. *(1) Short-chain Fructooligosaccharides (scFOS, derived from sucrose).*—Used to identify GF_3 and GF_4 (which will be the second and third/last major peaks present). This commodity is a representative of a category 1 fructan. Alternately one may use discrete GF_3 and GF_4 reagents (e.g., Wako Part No. 295-73401).

(2) Beneo Orafti HP Inulin.—Used to identify category 3 type fructans.

D. Preparation of Standards and Solutions

(a) *Fructan RT marker standards.*—Weigh 0.4 ± 0.004 g of each fructan reference material, scFOS (or ~0.050 g each of individual GF_3 and GF_4 standards) and Beneo Orafti HP. Transfer to a 1000 mL volumetric flask. Bring to volume with laboratory water and mix to ensure complete dissolution. Transfer ~1 mL of each to autosampler vials. Store at –20°C for up to 6 months.

(b) *Mobile phase A.*—Deliver laboratory water to an acceptable container and sparge with UHP helium for 10 min. Store under ~3–5 psi blanket of helium on the instrument. Expiration is 30 days at room temperature.

(c) *Mobile phase B.*—Deliver 1000 mL laboratory water to an acceptable container and sparge with UHP helium for 10 min. Add 40 ± 0.1 g of 50% (w/w) NaOH and continue to sparge for 2 additional min. Store under ~3–5 psi blanket of helium on the instrument. Expiration is 30 days at room temperature.

(d) *Mobile phase C.*—Deliver 1000 mL laboratory water to an acceptable container and sparge with UHP helium for 10 min. Add 40.8 ± 0.1 g sodium acetate trihydrate and continue to sparge for 5 additional min (or until dissolved). Then filter the solution through the membrane filter. Store under ~3–5 psi blanket of helium on the instrument. Expiration is 14 days at room temperature.

E. Sample Preparation

(a) *Powder sample.*—Reconstitution (if needed). Accurately weigh 5.0 ± 0.025 g into a 100 mL plastic beaker [record powder weight (PW)]. Tare and deliver 40 ± 0.2 g laboratory water to beaker [record water weight (WW)]. Allow to stir for 30 min, or until dissolved.

(b) *Sample dilution.*—Samples require differing dilutions according to their individual fructan content as per Table **2016.06D**. For example, a liquid ready-to-drink (RTD) sample containing 0.5% fructan would be diluted at a rate of 7 g to 200 mL. Record all weights to 4 decimal places. This solution can be used also with the quantitative fructan methodology in Part II. This is SW_1.

(c) *Filtration.*—After bringing the samples to volume, filter through a 0.45 µm nylon syringe filter into an autosampler vial (prepared samples can be stored in vials at 2–10°C for 5 days).

F. Instrumental Analysis

(a) *Gradient.*—Fructans are eluted using a gradient of NaOH and sodium acetate at 1.0 mL/min as per Table **2016.06E**. Column and detector compartment are maintained at 20°C. *Note:* A CarboPac PA1 guard is used for this procedure rather than the borate trap used in Part II for quantitative fructan determination. Therefore, if same day analysis is desired, two separate HPAEC-PAD systems are needed.

The gradient program recommended in Table **2016.06E** has

Table 2016.06E. Qualitative ID (Part I) gradient

Time, min	Flow, mL/min	A (laboratory water), %	B (500 mM NaOH), %	C (300 mM NaOAc), %	Curve
0	1.0	67.0	8.0	25.0	NA
0	1.0	67.0	8.0	25.0	5
40.0	1.0	0.0	8.0	92.0	5
45.0	1.0	0.0	8.0	92.0	5
45.1	1.0	67.0	8.0	25.0	5
55.0	1.0	67.0	8.0	25.0	5

Table 2016.06F. PAD waveform

Time, s	Potential, V	Integration
0.00	+0.05	
0.20	+0.05	Begin
0.40	+0.05	End
0.41	+0.75	
0.60	+0.75	
0.61	–0.15	
1.00	–0.15	

proven adequate for the qualitative determination required here, including for matrices containing maltodextrins (which tend to be the most problematic, in particular with respect to determining presence of GF_3 and/or GF_4). If necessary, it is acceptable to modify the Part I separation conditions to optimize resolution.

(b) *Electrochemical detector parameters.*—This method utilizes the carbohydrate triple waveform per Table **2016.06F**. *Note*: This waveform is not appropriate for disposable gold electrodes. The reference electrode is set to AgCl mode.

(c) *Injection.*—Make a single 4 µL injection of each sample test solution. At the start of each sequence, make six equilibration injections (one may use laboratory water or extra standards for the equilibration injections) to ensure that system is stable, followed by the two retention time standards. Bracket samples with standards after every six injections. Maintain autosampler sample compartment at 10°C.

G. Data Interpretation and Assignment of Commodity Factors (CF)

Using chromatograms from analysis of the RT standards for reference, determine whether GF_3, GF_4, and higher DP fructan forms (evidenced by peaks after ~36 min) are present in the test sample(s). Assign commodity factors based on the following rules:

(1) Presence of either GF_3 and/or GF_4, but no fructan peaks after ~36 min.—CF = 1.233.

(2) Presence of either GF_3 and/or GF_4, along with fructan peaks after ~36 min.—CF = 1.068.

(3) Absence of GF_3 and GF_4 and presence of fructan peaks after ~36 min.—CF = 0.9526.

Discrete ingredient commodity examples are shown in Figures **2016.06A–C**. Oligofructose containing materials derived from the partial hydrolysis of chicory inulin often display a characteristic peak with significant tailing at ~5 min, as illustrated in Figure **2016.06B**. If identification of fructan type is difficult, it may be helpful to also inject a selection of different commodities for direct comparison.

Part II – Quantitative Determination of Total Fructan

H. Apparatus and Materials

(a) *LC system.*—One biocompatible gradient pump; biocompatible refrigerated autosampler capable of injecting 4 µL; electrochemical detector with gold electrode capable of pulsed amperometric detection. For example, Dionex ICS-3000 or 5000 consisting of an SP, DP, WPS-3000 (TB; P or S type), and an electrochemical cell with a conventional gold working electrode and combination pH Ag/AgCl reference electrode or equivalent.

(b) *Analytical column set.*—Analytical column, Thermo CarboPac PA1, 250 × 4 mm; borate trap (replaces guard column), Thermo Part No. 047078 or equivalent.

(c) *Analytical balance.*—Readable to 0.01 mg.

(d) *pH meter.*—Readable to ±0.01.

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

(f) *Syringe filter.*—0.45 µm nylon.

(g) *Volumetric flasks.*—Glass, Class A, assorted sizes.

(h) *Volumetric pipets.*—Glass, Class A, assorted sizes.

(i) *Centrifuge tube.*—5 mL plastic (or greater, extra volume allows for room to neutralize excess reagent).

(j) *Microcentrifuge tubes.*—Assorted sizes.

(k) *Polypropylene beaker.*—100 mL.

(l) *Screw capped vials.*—No metal lined caps.

(m) *Graduated cylinders.*—Glass, assorted sizes.

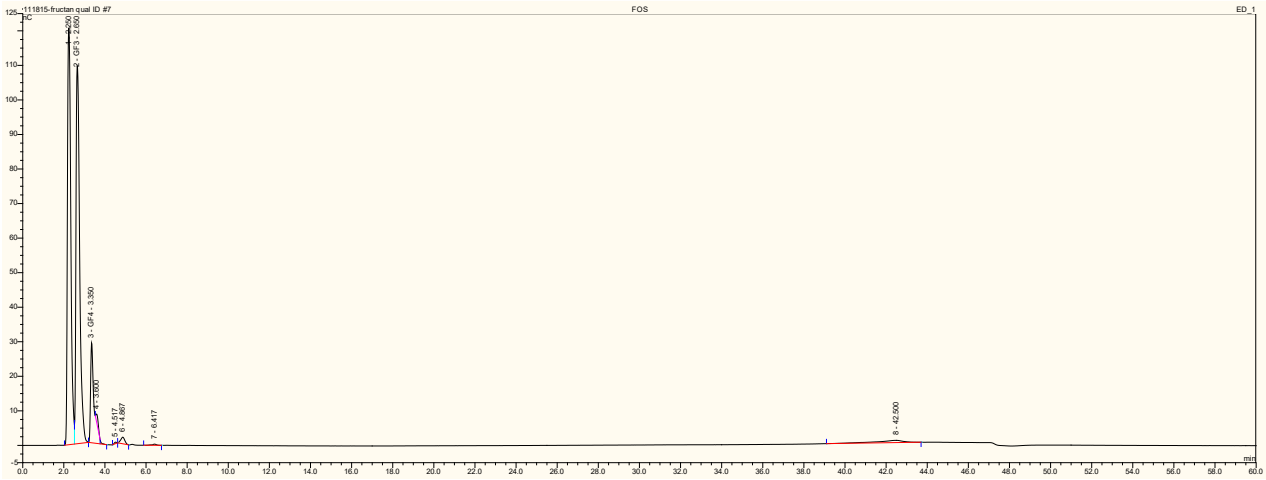

Figure 2016.06A. scFOS chromatogram (Part I).

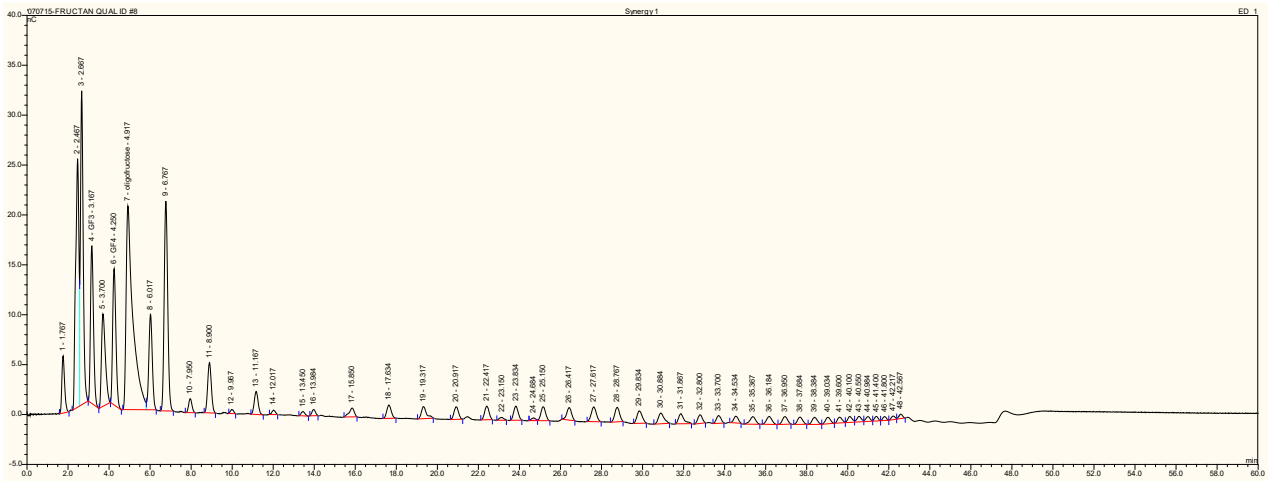

Figure 2016.06B. Beneo Orafti Synergy 1 chromatogram (Part I).

(n) *Desiccator and indicating Drierite (or equivalent) desiccant.*

(o) *Eluent filtration apparatus with disposable membrane filter.*—0.2 µm PES.

(p) *Micropipettor and tips.*—Assorted sizes.

(q) *Reagent bottles.*—Glass and plastic, assorted sizes.

(r) *Beakers.*—Glass, assorted sizes.

(s) *Helium sparge.*—Tubing and frit assembly.

I. Reagents

(a) *Laboratory water.*—ASTM Type 1 water.

(b) *NaOH solution.*—50%, w/w (Fisher Part No. SS254-500 or equivalent). If an alternate vendor is selected, it is imperative that the carbonate level be equivalent to or less than Fisher (≤0.10%).

(c) *NaOH solution.*—1 M.

(d) *Sodium acetate trihydrate.*—Reagent grade.

(e) *Glucoheptose.*—Sigma Part No. 71188 or equivalent. *Note*: This reagent has been discontinued by Sigma. Alternate suppliers include: Carbosynth (Part No. MG05213), MP Biomedicals (Part No. 05207893), and Cedarlane Labs (Part No. 157755 or 0520789380).

(f) *Fructose.*—Reference grade.

(g) *Sodium borohydride.*—>98% purity.

(h) *Maleic acid.*—>99.0% purity.

(i) *Acetic acid.*—Glacial, ACS.

(j) *Fructanase.*—Megazyme Part No. E-FRMXPD, or equivalent.

(k) *Sucrase.*—Megazyme Part No. E-SUCR. Demonstration of equivalent absence of activity toward fructan material is required for any substitutions.

J. Preparation of Standards and Solutions

(a) *Fructose stock solution 100 µg/mL.*—Weigh 0.05 ± 0.0025 g fructose reference material and transfer to a 500 mL volumetric. Bring to volume with laboratory water.

(b) *Glucoheptose IS.*—Weigh 0.1 ± 0.005 g of glucoheptose and transfer to a 100 mL volumetric. Bring to volume with laboratory water. It is recommended to portion out aliquots of this solution to avoid multiple freeze thaw cycles and extend useful life of reagent. Store frozen at –20°C for up to 6 months.

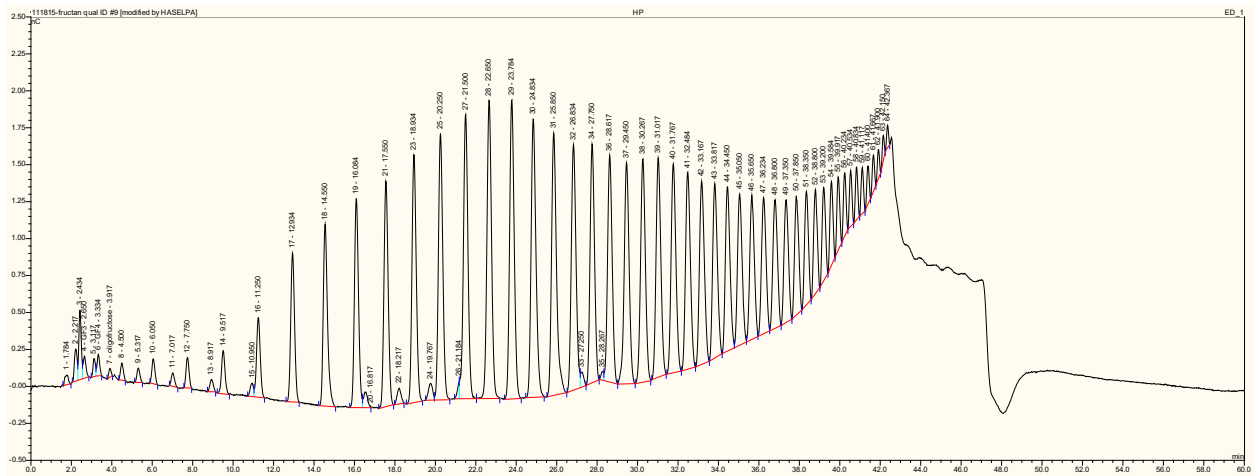

Figure 2016.06C. Beneo Orafti HP chromatogram (Part I).

Table 2016.06G. Quantitative fructan determination (Part II) gradient

Time, min	Flow, mL/min	A (lab water), %	B (500 mM NaOH), %	C (300 mM NaOAc), %	Curve
0	1.0	90.0	10.0	0.0	NA
0	1.0	90.0	10.0	0.0	5
20.0	1.0	90.0	10.0	0.0	5
20.1	1.0	0.0	80.0	20.0	5
30.0	1.0	0.0	80.0	20.0	5
30.1	1.0	90.0	10.0	0.0	5
45.0	1.0	90.0	10.0	0.0	5

(c) *Working standards (WS).*—Dilute fructose stock and glucoheptose IS solution with laboratory water, using class A glass pipets for transfer of fructose and IS solutions to appropriate volumetric flasks for final dilution, as follows: *(1) WS1*.—5 mL Fructose stock, 10 mL IS solution to 200 mL final volume.

(2) WS2.—5 mL Fructose stock, 5 mL IS solution to 100 mL final volume.

(3) WS3.—25 mL Fructose stock, 5 mL IS solution to 100 mL final volume

(4) WS4.—50 mL Fructose stock, 5 mL IS solution to 100 mL final volume.

Store frozen at –20°C for up to 6 months.

(d) *Acetic acid solution 0.2 M*.—Transfer 2.9 mL acetic acid to a 250 mL flask containing ~100 mL laboratory water. Dilute to volume with laboratory water and mix by inversion. Transfer to a suitable container for storage up to 2 years.

(e) *50 mM NaOH solution.*—Transfer 5 mL of 1 M NaOH into a 100 mL flask containing ~50 mL laboratory water. Bring to volume with laboratory water mix thoroughly. Store in suitable plastic container for up to 2 years.

(f) *Alkaline borohydride solution ~10 mg/mL.*—Immediately before use, weigh 100 mg sodium borohydride into a 15 mL tube. Dissolve in 5 mL of 50 mM NaOH (this is enough for 24 samples). Use for up to 4 h after addition of hydroxide.

(g) *Sodium maleate buffer 0.1 M, pH 6.5.*—Weigh 2.9 ± 1% of maleic acid in a 250 mL beaker. Dissolve in ~150 mL laboratory water. Adjust pH to 6.5 with 1 M NaOH. Transfer to 250 mL flask; bring to volume with laboratory water and mix thoroughly.

(h) *Sucrase solution (~30 units/mL).*—Measure 10 mL maleate buffer in a graduated cylinder. Deliver to sucrase vial. Cap and swirl gently to dissolve. Divide into ~450 µL aliquots and store frozen at –20°C (each tube is enough for two samples) for up to a year.

(i) *Acetate buffer.*—Combine 2.9 mL glacial acetic acid with 450 mL laboratory water. Adjust pH to 4.5 with 1 M NaOH. Bring total volume to 500 mL with laboratory water and mix thoroughly.

(j) *Fructanase solution (~909 units/mL).*—Dissolve contents of fructanase vial in 22 mL acetate buffer. Swirl gently to dissolve. Divide into ~1 mL aliquots and store frozen at –20°C for up to a year (each tube should contain enough for nine samples).

(k) *Mobile phase A.*—Deliver laboratory water to an acceptable container and sparge with UHP helium for 10 min. Store under ~3–5 psi blanket of helium on the instrument. Expiration is 30 days at room temperature.

(l) *Mobile phase B.*—Deliver 1000 mL laboratory water to an acceptable container and sparge with UHP helium for 10 min. Add 40 ± 0.1 g of 50% (w/w) NaOH and continue to sparge for 2 additional min. Store under ~3–5 psi blanket of helium on the instrument. Expiration is 30 days at room temperature.

(m) *Mobile phase C.*—Deliver 1000 mL laboratory water to an acceptable container and sparge with UHP helium for 10 min. Add 40.8 ± 0.1 g sodium acetate trihydrate and continue to sparge for 5 additional min (or until dissolved). Then filter the solution through the membrane filter. Store under ~3–5 psi blanket of helium on the instrument. Expiration is 14 days at room temperature.

K. Sample Preparation

If running in parallel with Part I, above, an aliquot from the diluted solution, **E(b)**, can be used in this method. If so skip to step **(c)**, below. Otherwise proceed to step **(a)**, below

(a) *Powder sample reconstitution (if sample is RTF, skip this step).*—Accurately weight 5.0 ± 0.025 g into a 100 mL plastic beaker and record weight. This is PW. Tare and deliver 40 ± 0.2 g laboratory water to beaker. Record water weight. This

Table 2016.06H. Column cleaning/trap regeneration gradient

Time, min	Flow, mL/min	A (Lab water), %	B (500 mM NaOH), %	C (300 mM NaOAc), %	Curve
0	1.0	60.0	40.0	0.0	NA
0	1.0	60.0	40.0	0.0	5
15.0	1.0	60.0	40.0	0.0	5
15.1	1.0	0.0	40.0	60.0	5
30.0	1.0	0.0	40.0	60.0	5
30.1	1.0	60.0	40.0	0.0	5
45.0	1.0	60.0	40.0	0.0	5

is WW. Allow to stir for 30 min, or until dissolved/uniformly suspended.

(b) *Sample dilution.*—As with the qualitative analyses, samples require different dilutions according to their individual fructan content (Table **2016.06D**). Record all weights to 4 decimal places. This is SW_1.

(c) *Removal of inherent glucose, fructose, and sucrose.*—Transfer 0.2 g ± 10% of the diluted sample from step **E(b)** or **K(b)** above, as appropriate (as per guidelines in Table **2016.06D**) to a glass screw cap scintillation vial and record weight to 4 decimal places. This is SW_2. Add 200 µL of sucrase solution, **J(h)**, to vial. Cap, swirl gently, and incubate at 40°C for 2 h (do not use foil lined caps). After the sucrase incubation, add 700 µL laboratory water to the scintillation vial. Then add 200 µL sodium borohydride solution, **J(f)**. Cap, swirl, and incubate at 40°C for 1 h. After the borohydride reduction is complete, neutralize the excess reagent with 500 µL of 0.2 M acetic acid, **J(d)**. Swirl gently (leave uncapped to allow gas generated to vent safely) and allow samples to sit at room temperature for 15 min.

Note: Gas bubble formation after the addition of $NaBH_4$, but prior to addition of acetic acid, may be a sign of improper sample pH and will negatively impact final results. If this is observed, further investigation of sucrase buffer and/or 50 mM NaOH solution is recommended to ensure the borohydride reagent is sufficiently basic to remain stable.

(d) *Fructan hydrolysis.*—Add 100 µL glucoheptose IS solution, **J(b)**, and 100 µL fructanase solution, **J(j)**. Cap, swirl, and incubate at 40°C for 30 min. After the incubation is completed, swirl gently to ensure a homogeneous sample and filter through a 0.45 µm nylon syringe filter into autosampler vials (prepared samples can be stored in vials at 2–10°C for 5 days).

L. Instrumental Analysis

(a) *Gradient.*—Fructose and glucoheptose are eluted isocratically using 50 mM NaOH at 1.0 mL/min for 20 min. The column is then washed for 10 min with 400 mM NaOH and 60 mM sodium acetate. Following the wash step, the column is re-equilibrated with 50 mM NaOH for 15 min (Table **2016.06G**). Column and detector compartment are maintained at 20°C. It is recommended to clean the column and trap approximately every five quantitative analytical sequences, with three complete cycles of the conditions outlined in Table **2016.06H**. [Five sequences would roughly equate to ~130 sample and/or control injections. Failure to regularly clean the column/trap set may result in breakthrough of borate to the analytical column degrading method performance (primarily observed in the peak asymmetry of the glucoheptose).]

(b) *Injection.*—Make a single 4 µL injection of each sample test solution. At the start of each sequence, make at least six equilibration injections to ensure that system is stable (note that an observed calibration error in excess of 5% on average for a level of standard may be indicative of insufficient equilibration time), followed by the four levels of WS. Bracket samples with WS after every 13 injections. Maintain autosampler at 10°C.

(c) *Electrochemical detector parameters.*—This method utilizes the carbohydrate triple waveform per Table **2016.06F**. *Note*: This waveform is not appropriate for disposable gold electrodes. The reference electrode is set to AgCl mode.

(d) *Retention times.*—Typically fructose elutes around 10–11 min and glucoheptose around 15–18 min (*see* Figures **2016.06D** and **E**).

M. Calculations

(a) *Fructose stock standard.*—Calculate the fructose stock concentration according to:

$$\text{Fructose concn, µg/mL} = \frac{\text{fructose wt. (g)} \times 1\,000\,000\ \text{µg/g} \times \text{purity}}{500\ \text{mL}}$$

where purity = %purity [from the Certificate of Analysis (CoA)/100%].

(b) *WS.*—Calculate WS concentrations (µg/mL) as follows:

$$\text{WS4} = \text{Fructose stock} \times 50/100$$

$$\text{WS3} = \text{Fructose stock} \times 25/100$$

$$\text{WS2} = \text{Fructose stock} \times 5/100$$

$$\text{WS1} = \text{Fructose stock} \times 5/200$$

(c) *Calibration.*—Obtain the peak areas for fructose and

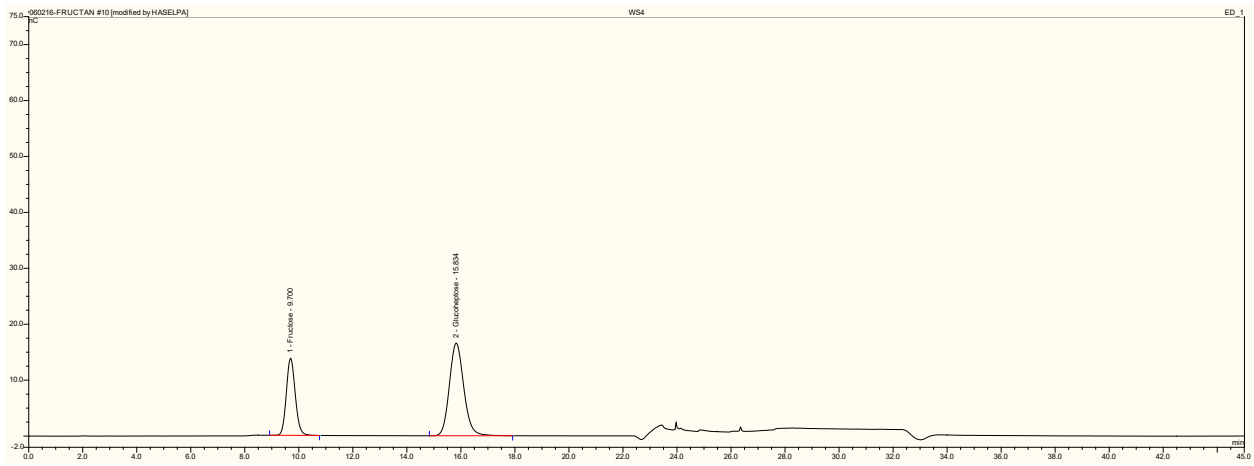

Figure 2016.06D Working standard (WS4) chromatogram (Part II).

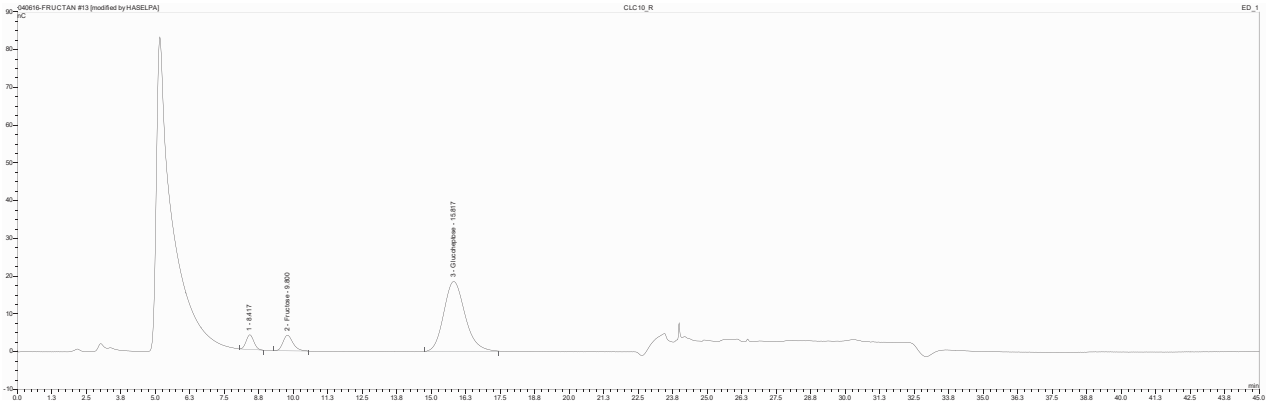

Figure 2016.06E. Control powder, milk based, chromatogram (Part II).

glucoheptose for each level of working standard. Fit the peak area ratios (responses) and corresponding standard concentrations, in µg/mL, to a quadratic model. Do not force intercept to zero. Identify glucoheptose as the internal standard.

(d) *Sample weight factors (SWF).*—Calculated as follows:

(1) RTF products and commodities.

$$SWF = SW1\ (g) \times SW2\ (g) \times 10\,000$$

(2) Powder products.

$$SWF = \frac{PW\ (g)}{PW\ (g) + WW\ (g)} \times SW1\ (g) \times\ SW2\ (g) \times\ 10\,000$$

where SW_1 = weight of material delivered to a volumetric flask (RTF, or reconstituted powder) and diluted as per sample table guidelines. For example, an RTF product of 0.21–1.0% fructan would have an SW_1 of 7 g (±10%) into a 200 mL volumetric flask. SW_2 = weight of solution from volumetric flask per sample table guidelines (volumetric dilution of SW_1). Value should be 0.2 g ± 10%. PW = weight of powder product weighed to be reconstituted (5 g ± 10%). WW = weight of water used to reconstitute powder products (40 g ± 10%).

(e) *Dilution factor (DF).*—Calculated as follows:

(1) Low-level fructan (RTF 0.03–0.2%, powder 0.27–1.0%).

DF = 50 g × 2 mL

(2) Mid-level fructan (RTF 0.21–1.0%, powder 1.1–10%).

DF = 200 g × 2 mL

(3) High-level fructan (RTF 1.1–5.0%, powder 11–45%).

DF = 500 g × 2 mL

(4) Commodity DF (46–100%).

DF = 1000 g × 2 mL

(f) *Commodity factors (CF).*—As determined, based on analysis in Part I. The factors chosen for the three groups listed in section **G**, in Part I, were done in such a way so as to cover the spectrum of fructan commodities currently in use. Any factor should then impart a bias of no more than ~5% for the grouping that it covers.

If the average DP of the fructan is known, this factor can be explicitly calculated according to:

$$CF = \left(\frac{DP_{Avg}}{DP_{Avg} - 1}\right) * \left(0.9 + \frac{0.1}{DP_{Avg}}\right)$$

(g) *Sample total fructan (g/100 g).*—Interpolate the concentration of fructose in the injected sample (µg/mL) using the corresponding fructose response parameters. Calculate the total fructan concentration using the proper weights and factors:

$$Total\ Fructan\left(\frac{g}{100g}\right) = \frac{Interpolated\ Fructose\ x\ DF\ x\ CF}{SWF}$$

where DF = dilution factor; CF = commodity factor; SWF = sample weight factor.

Validation Protocol

Linearity

A total of 43 standard curves (two bracketed sets of standards for each curve) were collected during the course of

Table 1. Recovery from spiked in-house GOS containing placebo

Sample type	No. of replicates[a]	Spiking material[b]	Nominal 0.3% spike		Nominal 3% Spike	
			Avg. % rec.	RSD, %	Avg. % rec.	RSD, %
Infant formula powder, low lactose milk based with GOS	9	scFOS	100	5.43	96.2	2.85
Infant formula powder, low lactose milk based with GOS	9	Oligofructose	99.0	6.09	96.0	2.35
Infant formula powder, low lactose milk based with GOS	9	Oligofructose/inulin	98.4	2.85	97.2	2.04

[a] Triplicates on each of 3 days.

[b] In-house material. scFOS = Ingredion Nutraflora FOS powder; oligofructose = Beneo Orafti P95 powder; oligofructose/inulin = Beneo Orafti Synergy 1.

Table 2. SPIFAN kit products without added fructan - (analyzed for specificity evaluation)

Sample type, batch	Total fructan found[a]
Infant elemental powder	ND[b]
Adult nutritional RTF high protein	ND
Adult nutritional RTF high fat	ND
Infant formula RTF milk based	ND
SRM 1849a	ND
Infant formula powder partially hydrolyzed milk based	ND
Infant elemental powder	ND
Infant formula powder milk based	ND
Infant formula powder soy based	ND
Infant formula RTF milk based	ND
Adult nutritional RTF high protein	ND

[a] Mean fructan level detected in Part II of method analysis.

[b] ND = Not detected; No fructose peak could be identified. Detection limit was not experimentally determined but it is estimated that apparent fructan in these samples is consistently <0.01%.

the validation work, spanning a period of 10 months. Relative calibration errors for each standard were calculated and used as the basis for determining adequacy of fit to the quadratic calibration model.

Accuracy

Because there are no relevant certified reference materials, assessment of accuracy was based entirely on recovery of fructans from spiked samples. The samples used for these experiments were products included in the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Sample Kit. Of these, six were determined to contain fructan in the form of either scFOS or oligofructose (group 1 fructan, for purposes of CF assignment). These six samples were overspiked with either scFOS or oligofructose at ~50 and 100% of the endogenous level. Two of the unfortified SPIFAN products were spiked at two levels with either inulin (HP) or an oligofructose/inulin mixture (Synergy 1). SPIFAN sample overspike and spike configurations are described in Tables **2016.06B** and **C**. Both of these sample sets were analyzed in duplicate on each of 3 days. In addition to the SPIFAN matrixes, in-house low-lactose, milk-based infant formula containing GOS was spiked at two levels with three different fructans. Table 1 provides spike information for the latter.

Precision

The six fructan-containing SPIFAN samples were analyzed in duplicate on each of 6 days. In addition to the SPIFAN products, four Abbott in-house materials were also analyzed. Three of those were analyzed in duplicate on each of 10 days, by each of two independent laboratories. A fourth in-house (control) sample was analyzed in duplicate on each of 10 days by three independent laboratories. Repeatability and intermediate precision metrics were calculated for each of the 10 samples for which replicate data was collected.

Specificity

Specificity was evaluated primarily by analysis of products not fortified with fructans, in order to determine potential apparent background (Table 2). Each of these products was analyzed in duplicate. In addition, chromatograms of multiple maltodextrin commodities and products containing either hydrolysate protein or free amino acids were examined for potential interferences

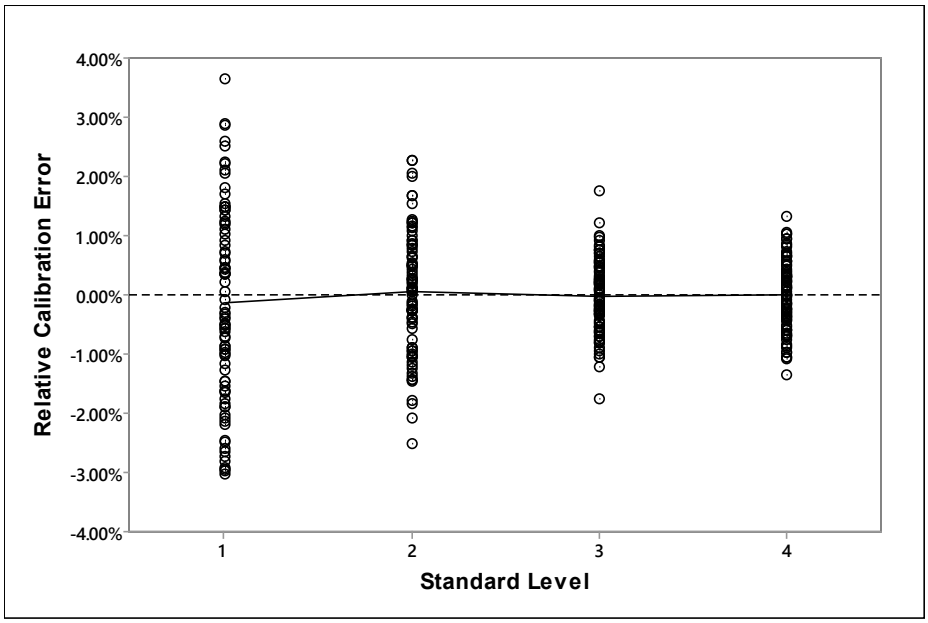

Figure 2. Relative calibration errors (composite from 43 analytical runs).

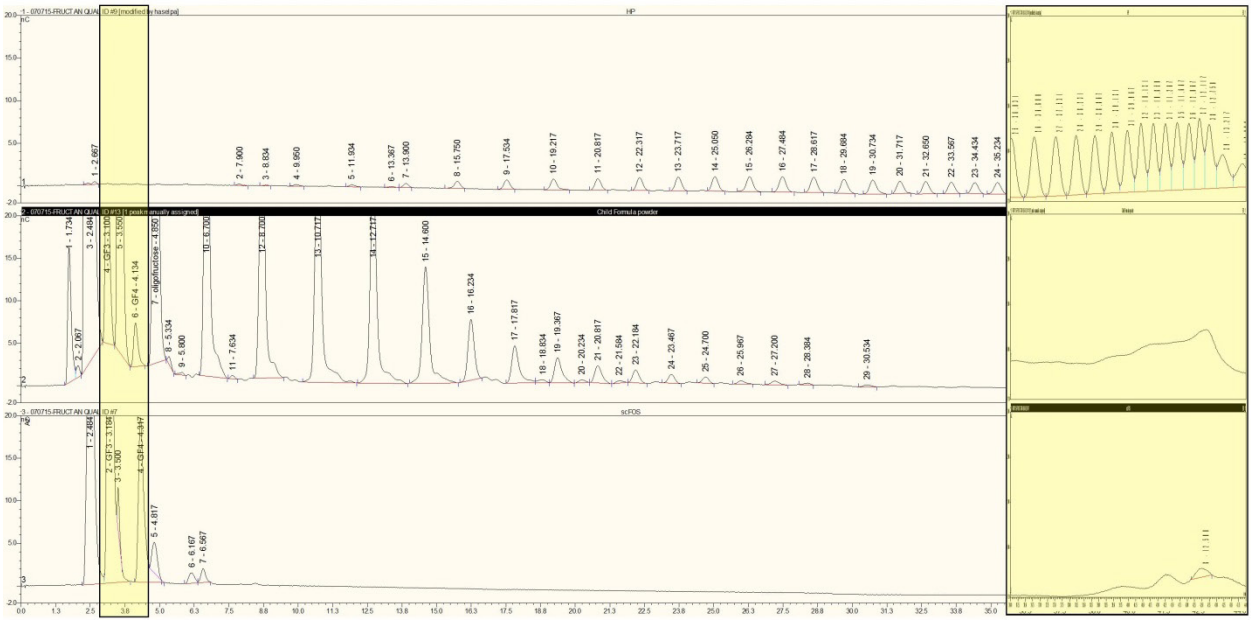

Figure 3. Example of product containing scFOS at ~0.28 g/100 g (Part I) – Upper trace: inulin (Beneo Orafti HP); middle trace: SPIFAN child formula powder, 00866RF00; lower trace: Ingredion Nutraflora scFOS. Time interval from 36–44 min zoomed in to show detail.

that could complicate CF assignment following the guidance for Part I analysis.

Minimum Detection Limit (MDL)/LOQ

The LOQ was empirically demonstrated by analysis of a low-level spike solution, also containing a high level of sucrose. scFOS was spiked into laboratory water at a concentration of 0.03 g/100 g. Sucrose was added to this sample at a concentration of 9 g/100 g. This solution was analyzed in triplicate on each of 3 days. Average recovery, repeatability, and intermediate precision were calculated from this data.

Results and Discussion

Linearity

A graphical summary of the relative errors for each standard from the 43 calibration data sets is presented in Figure 2. These results indicate that, consistent with expectations, uncertainty (as evidenced by the dispersion) increases as concentration decreases. However there is no significant evidence of a concentration dependent bias and relative calibration errors are less than 4% for every individual standard. Consequently, it can be concluded that fit of calibration data to the quadratic model is consistently acceptable.

Accuracy (Spike Recovery)

Results of the various recovery experiments are summarized in Tables **2016.06B**, **2016.06C**, and Table 1. Average recoveries ranged from 92.9 to 108%. The lowest recoveries (92.9 and 94.2%) were obtained for scFOS from the SPIFAN adult Nutritional RTF high-fat product, while the highest (108 and 105%) were for oligofructose from the SPIFAN toddler formula powder, milk based. All of the average recoveries were with the acceptable range of 90–110%, as specified in SMPR 2014.002.

Precision

Precision metrics are summarized in Table **2016.06A**. Repeatability RSDs (RSD_r) ranged from 1.09 to 3.67%, all well below the requirement of 6%. Intermediate precision RSDs (RSD_{IP}) ranged from 2.57 to 6.79%. Intermediate precision requirements are not explicitly called out in SPIFAN SMPRs, however, in all but one case, intermediate precision performance met the stated requirement for repeatability.

Specificity

As indicated in Table 2, no detectable fructose was found in any of the unfortified SPIFAN products when they were subjected to quantitative testing (Part II) for total fructan content. Because of the diversity of these matrixes, including carbohydrate systems, this is good evidence of the ability to selectively detect fructans (as enzymatically released fructose).

Example chromatograms relevant to the qualitative Part I analysis for CF assignment are shown in Figures 3–5. Maltodextrin-containing matrixes tend to be potentially the most problematic, especially with respect to assessing the presence or absence of GF_3 and/or GF_4. It is important, though, to recognize that this step is not a screening tool to confirm whether or not fructans are present. It is intended only to provide sufficient information to allow selection of the most appropriate correction factor. The quantitative determination of enzymatically released fructose not only provides the basis for

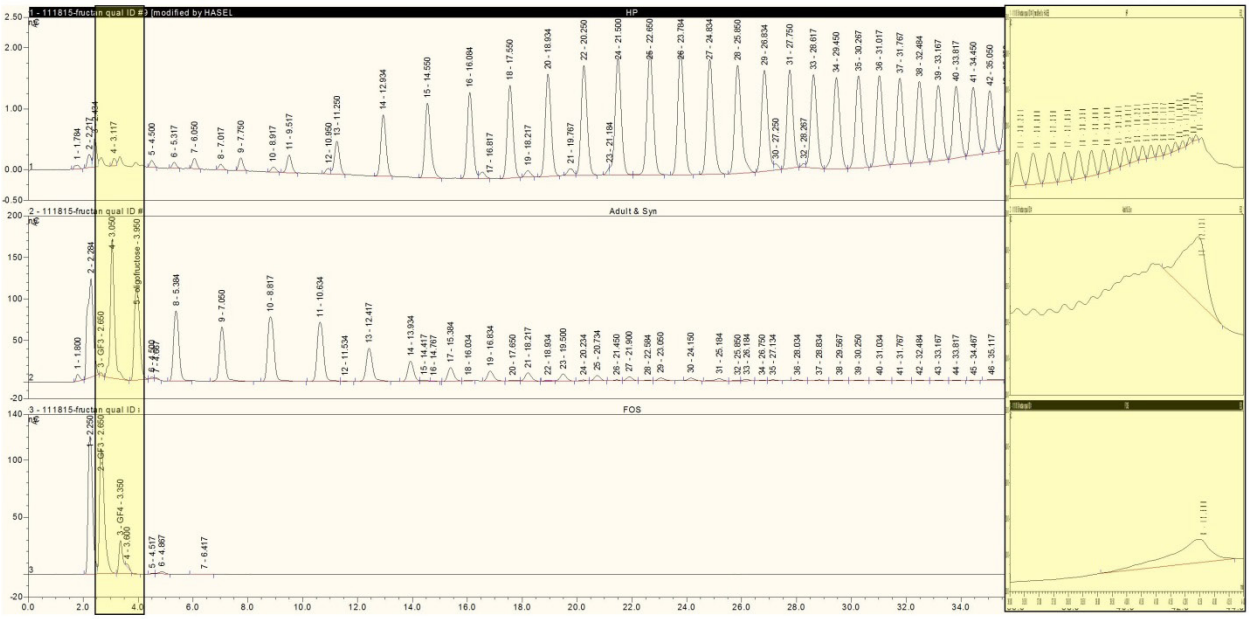

Figure 4. Example of product containing Synergy 1 spiked at ~1.1 g/100 g (Part I), – Upper trace: inulin (Beneo Orafti HP); middle trace: SPIFAN adult nutritional powder low fat, 00859RF00; lower trace: Ingredion Nutraflora scFOS. Time interval from 36–44 min zoomed in to show detail.

calculation of the actual fructan content but, at the same time, serves as the primary determinant of selectivity.

LOQ

Data relevant to LOQ is summarized in Table 3. For the 9% sucrose solution spiked with 0.03% scFOS, average recovery was 97%, RSD_r was 6.12%, and RSD_{IP} was 7.42%. This RSD_r at this level is just beyond the SMPR limit of 6%. Table 3 also shows data for the SPIFAN infant formula powder with FOS/GOS. The total fructan content of this material was determined to be very close to the SMPR specified LOQ of 0.03%. For that sample, both RSD_r (2.14%) and RSD_{IP} (3.19%) were well below the 6% repeatability limit.

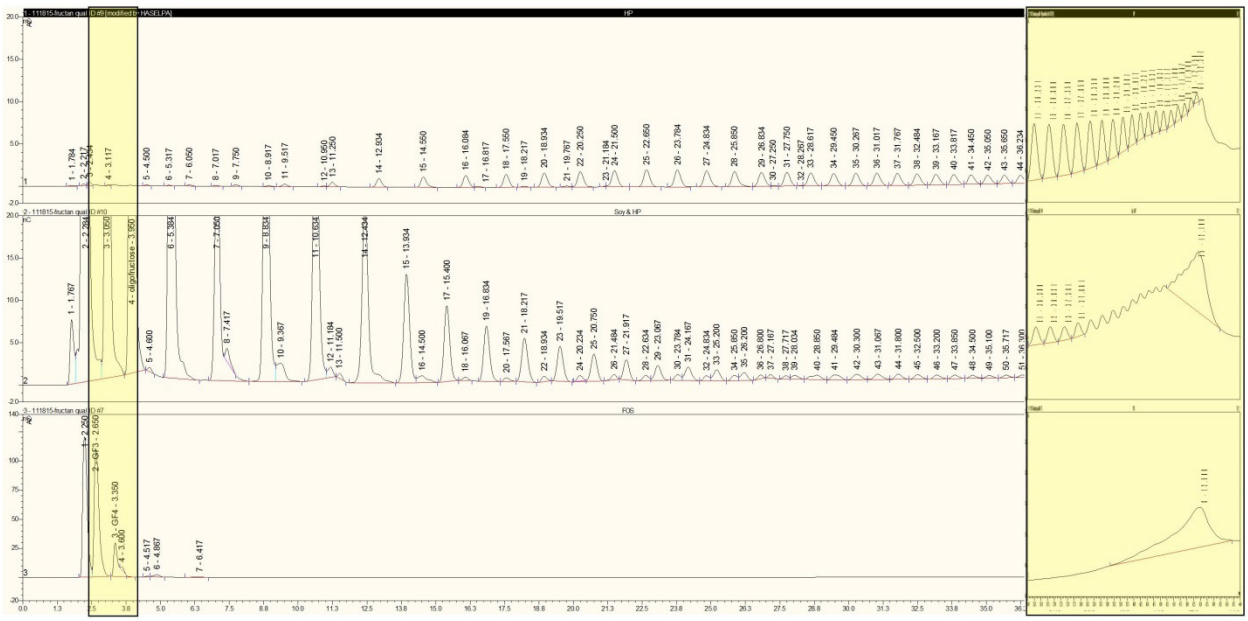

Figure 5. Example of product containing HP (Part I), spiked at ~1.1 g/100 g – Upper trace: inulin (Beneo Orafti HP); middle trace: SPIFAN infant formula powder partially hydrolyzed soy, 410457651Z; lower trace: Ingredion Nutraflora scFOS. Time interval from 36–44 min zoomed in to show detail.

Table 3. Data for evaluating LOQ

Sample type	No. of replicates	Mean (g/100 g, RTF)	SD_r	RSD_r, %	SD_{IP}	RSD_{IP}, %
0.03% FOS spike in 9% sucrose solution[a]	9[b]	0.029	0.0018	6.12	0.0021	7.42
Infant formula powder with FOS/GOS[c]	12[d]	0.036	0.0008	2.14	0.0012	3.29

[a] No additional matrix, solution in water.
[b] Triplicates on each of 3 days.
[c] Material from SPIFAN sample kit.
[d] Duplicates on each of 6 days.

Conclusions

Performance metrics from the validation work indicate that the method conforms to the range, LOQ, precision, and recovery requirements established in SMPR 2014.002. In addition, specificity appears to be adequate, as evidenced by the failure to detect any apparent fructan in the unfortified samples that were analyzed. Common carbohydrate ingredients, GOS and various maltodextrins, although producing more complicated chromatograms for interpretation, do not generally create undue difficulty for assignment of CFs in the qualitative analysis. We conclude that the method is suitable for determination of inulin-type fructans in infant formulas and pediatric/adult nutritional products.

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Biotin in Infant, Pediatric, and Adult Nutritionals by High-Performance Liquid Chromatography and Fluorescence Detection: Single-Laboratory Validation, First Action 2016.11

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A reversed-phase HPLC method with postcolumn protein conjugation and fluorescence detection for the quantitative determination of biotin in infant, pediatric, and adult nutritionals was developed and evaluated in a single-laboratory validation (SLV). Sample of appropriate size is mixed with 2% metaphosphoric acid to precipitate out the protein. The filtrate is injected onto a C18 HPLC column in which biotin and riboflavin are separated with an appropriate mobile phase. The biotin, after eluting from the column, binds with the streptavidin fluorescein to become a fluorescent conjugate. The conjugate is then detected by fluorescence at λ_{ex} = 495 nm and λ_{em} = 518 nm. A column switch is used in the method as an option to shorten the run time from 30 to 15 min, by eluting out riboflavin at a higher flow rate. In this SLV, a total of 19 AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals matrixes representing a range of infant, pediatric, and adult formulas were evaluated for their biotin content. The analytical range was 1.66–142 µg/100 g reconstituted final product. The repeatability and intermediate precision ranged from 0.5 to 3.0% RSD_r and from 1.3 to 4.5% RSD_{iR}, respectively. Recovery from spiked matrixes varied from 95 to 111%, and accuracy of quantification using Standard Reference Material 1849a ranged from 99 to 105%. The LOQ in reconstituted product was estimated to be 0.8 µg/100 g. The method was approved by the Expert Review Panel as First Action at the 2016 AOAC INTERNATIONAL Mid-Year Meeting.

The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) developed *Standard Method Performance Requirements* (SMPRs®) for Biotin in Infant Formula and Adult/Pediatric Nutritional Formula and called for reference methods to determine total biotin in all forms (powders, ready-to-feed liquids, and liquid concentrates) of infant, adult, and/or pediatric formula for dispute resolution (1).

Biotin is a water-soluble vitamin also known as vitamin B_7 or vitamin H. It functions in important metabolic processes of carbohydrates, fats, and amino acids. Biotin is a monocarboxylic acid containing a cyclic urea structure with the sulfur atom in a thioether linkage; biocytin, the intermediate metabolite of biotin, is an amide formed from biotin and lysine (Figure 1).

This method is revised from a method published in 2006, in which SPE was used for sample preparation to remove riboflavin and HPLC with a fluorescence detector (FD) was used for the detection of the biotin/streptavidin/fluorescein conjugate formed during a postcolumn derivatization (2). In this new method, the SPE clean-up step is omitted from the sample preparation, and the revised sample preparation involves simple reconstitution, dilution with methanol/water, and protein precipitation with metaphosphoric acid. A column switch is used as an option to elute out riboflavin in a shorter run time.

Biocytin was subjected to the sample preparation condition and verified to be stable. It eluted out as a well-resolved peak from the biotin peak using this HPLC method. All of the 19 tested SPIFAN samples were found to be free of biocytin.

To find out the biotin and biocytin content from the potential inherent biotin conjugates and biocytin conjugates in the SPIFAN placebo formula, three ways of hydrolysis were attempted during sample preparation, adapting the protocols reported by Lahély et al. (3) and Höller et al. (4): (*1*) acidic hydrolysis with 2 N sulfuric acid in an autoclave at 120°C for 30 min; (*2*) enzymatic digestion in citric buffer with papain at 37°C for 16 h; and (*3*) acidic hydrolysis with 2 N sulfuric acid in an autoclave at 120°C for 30 min, followed by pH adjustment to 5.7, and enzymatic digestion in citric buffer with papain at 37°C for 16 h. All of the three tested SPIFAN placebo samples were found to be free of biotin conjugates or biocytin conjugates.

AOAC Official Method 2016.11
Biotin in Infant, Pediatric, and Adult Nutritionals
High-Performance Liquid Chromatography and Fluorescence Detection
First Action 2016

A. Principle

The basis of this method is the strong affinity between biotin and streptavidin. This method is applicable to infant, pediatric, and adult nutritional products. Samples of appropriate size are mixed with 2% metaphosphoric acid to precipitate out the

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This method was approved by the AOAC Expert Review Panel for SPIFAN Nutrient Methods as First Action.

The Expert Review Panel for SPIFAN Nutrient Methods 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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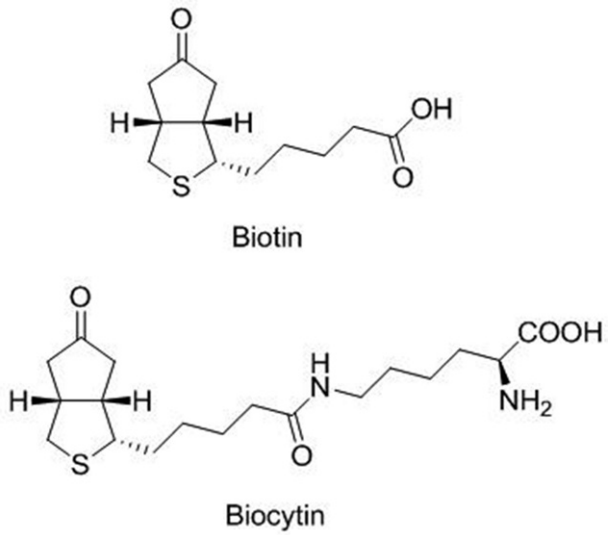


Figure 1. Chemical structures of biotin and biocytin.

proteins to produce a filtrate, which is subjected to subsequent HPLC analysis. The mobile phase (20% methanol in 0.02 M phosphate buffer at pH 7.0) is pumped at 0.4 mL/min. The postcolumn reagent (0.8 µg/mL streptavidin fluorescein in biotin mobile phase) is pumped at 0.2 mL/min. The biotin, after eluting from the column, binds with streptavidin fluorescein to become a fluorescent conjugate and is detected by an FD. The HPLC run takes 15 min for each injection when a column switch is used. The column switch allows the elution of riboflavin at 11 min, with a high flow rate of 1.5 mL/min. Without the column switch, the run takes 30 min for each injection, with the elution of riboflavin at 24 min.

B. Apparatus

Common laboratory equipment and, in particular, the following:

(a) *HPLC system*.—Two isocratic pumps; autosampler capable of injecting 20 µL; FD; high-pressure mixing tee; Aura's postcolumn knitted reactor coil: 15 m Teflon tubing (0.25 mm id, 1/16 in. od), 0.75 mL total volume, Cat. No. KRC 15-25, or equivalent; and reactor coil–compatible VALCO nuts and grooved ferrule, or equivalent. The system should be configured as shown in Figure **2016.11A** when the column switch is used as an option.

(b) *Cover*.—Adequate to keep the postcolumn reaction coil from light.

(c) *Analytical column*.—Agilent Poroshell 120 EC-C18, 4.6 × 50 mm, 2.7 µm, or equivalent.

(d) *Analytical balance*.—Capable of weighing to the nearest 0.00001 g.

(e) *pH Meter*.

(f) *Microcentrifuge*.—Capable of centrifuging at 10 000 × *g*.

(g) *Microcentrifuge tubes*.—2 mL.

(h) *Disposable syringe*.

(i) *Syringe filter*.—Nylon 0.45 µm or equivalent.

(j) *Filter assembly for filtering mobile phase*.

(k) *Nylon filter membrane for filtering mobile phase*.—0.45 µm or equivalent.

(l) *Graduated cylinders*.—Assorted sizes.

(m) *Disposable transfer pipets*.

(n) *Volumetric flasks*.—Class A, assorted sizes.

(o) *Volumetric pipets*.—Class A, assorted sizes.

(p) *Yellow lights or yellow shields with a cutoff of 385 nm*.

(q) *Magnetic stirring plate*.

(r) *Magnetic stirring bars*.

C. Chemicals and Reagents

(a) *Biotin standard*.—U.S. Pharmacopeia (USP) reference, official lot. Store per label instructions.

(b) *Ethanol (reagent alcohol)*.—American Chemical Society (ACS) or equivalent.

(c) *Streptavidin fluorescein conjugate*.—1 mg/mL, GeneTex Streptavidin–fluorescein isothiocyanate (Streptavidin-FITC; Cat. No. GTX30950) or AnaSpec Streptavidin-FITC (Cat. No. AS-60659-FITC), or equivalent. Store per label instructions.

(d) *Laboratory water*.

(e) *Methanol*.—HPLC grade or equivalent.

(f) *pH Meter buffer solutions for pH meter standardization*.

(g) *Metaphosphoric acid*.—ACS, 33.5–36.5%, or equivalent.

(h) *Sodium phosphate monobasic (SPM)*.—ACS or equivalent.

(i) *Sodium phosphate dibasic (SPD)*.—ACS or equivalent.

D. Standard and Solution Preparation

Note: Because biotin is light sensitive, all standards must be prepared, handled, and stored in the dark or under yellow-shielded lighting (*see* **B**), unless otherwise stated.

(a) *0.2 M SPM*.—Weigh 27.800 (±2.780) g SPM into a 1 L volumetric flask. Add about 800 mL laboratory water and mix until all solids dissolve. Dilute to volume with laboratory water. Mix well. Expiration: 1 month at room temperature.

(b) *0.2 M SPD*.—Weigh 28.390 (±2.840) g SPD into a 1 L volumetric flask. Add about 800 mL laboratory water and mix until all solids dissolve. Dilute to volume with laboratory water. Expiration: 1 month at room temperature.

(c) *0.02 M phosphate buffer (pH 7)*.—Place a 400 mL beaker on a stir plate. Using graduated cylinders, transfer 78 mL 0.2 M SPM and 168 mL 0.2 M SPD into the beaker. Adjust pH of the solution in the beaker to 7.00 (±0.05) using SPM and SPD. This solution is 0.2 M phosphate buffer (pH 7) and is made fresh for mobile phase preparation. Using a graduated cylinder, transfer 200 mL 0.2 M phosphate buffer (pH 7) into a 2 L volumetric flask. Dilute to volume with laboratory water and mix well. This solution is 0.02 M phosphate buffer (pH 7). Expiration: 1 week at room temperature.

(d) *Biotin mobile phase*.—Use a graduated cylinder to transfer 400 mL methanol into a 2 L volumetric flask. Dilute to volume with 0.02 M phosphate buffer (pH 7). Mix well, filter, and degas in a sonicator for 5 min. Transfer to a suitable mobile phase container. Expiration: 1 week at room temperature.

(e) *2% Metaphosphoric acid*.—Weigh 60 (±6) g of metaphosphoric acid (33.5–36.5%) into a 1 L beaker. Add about 800 mL laboratory water and mix until all solids dissolve. Quantitatively transfer the solution to a 1 L volumetric flask, dilute to volume with laboratory water, and mix well. Store refrigerated. Expiration: 1 week.

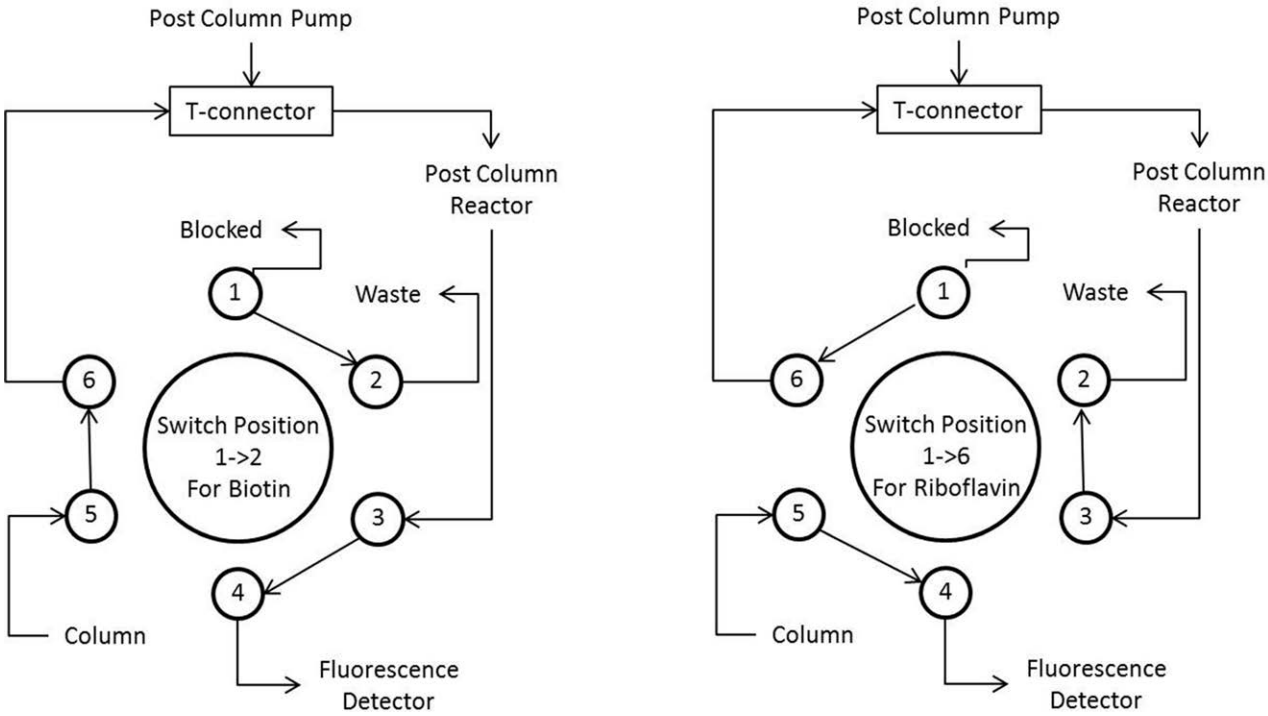


Figure 2016.11A. HPLC system configuration with a column switch.

(f) *50% Ethanol*.—Use a graduated cylinder to transfer 250 mL ethanol to a 500 mL volumetric flask. Dilute to volume with laboratory water. Expiration: 1 month at room temperature.

(g) *50% Methanol*.—Use a graduated cylinder to transfer 500 mL of methanol into a 1 L volumetric flask. Dilute to volume with laboratory water. Expiration: 1 month at room temperature.

(h) *Postcolumn reagent*.—Use a volumetric pipet to transfer 0.8 mL streptavidin fluorescein into a 1 L volumetric flask and dilute to volume with biotin mobile phase. Expiration: 1 week at room temperature.

(i) *Biotin stock standard solution (100 µg/mL)*.—Weigh 0.05000 (±0.0050) g USP biotin reference standard. Quantitatively transfer the standard to a 500 mL volumetric flask using 50% ethanol. Mix well. Transfer to a 500 mL amber bottle. Store refrigerated. Expiration: 3 months.

(j) *Biotin intermediate solution (1000 ng/mL)*.—Use a volumetric pipet to transfer 1 mL biotin stock standard solution into a 100 mL volumetric flask. Dilute to volume with laboratory water. Mix well. Expiration: make fresh on day of use.

(k) *Biotin working standards (5, 10, 20, 40, 60, 80, and 100 ng/mL)*.—For the 100 ng/mL working standard, use a volumetric pipet to transfer 5 mL biotin intermediate solution into a 50 mL volumetric flask. Dilute to volume with biotin mobile phase and mix well. For the 5, 10, 20, 40, 60, and 80 ng/mL working standards, use volumetric pipets to respectively transfer 0.5, 1, 2, 4, 6, and 8 mL of the 100 ng/mL working standard into 10 mL volumetric flasks. Dilute the volumetric flasks to volume with biotin mobile phase and mix well. Expiration: make fresh on day of use.

E. Sample Preparation

Note: Because biotin is light-sensitive, all samples must be prepared, handled, and stored in the dark or under yellow-shielded lighting (*see* **B**), unless otherwise stated.

(a) Accurately weigh up to 4 g powder product or up to 20 g liquid product in a 50 mL volumetric flask. The final concentration of biotin in the 50 mL solution should be in the range of 8–60 ng/mL. Add 20 mL water to reconstitute powder samples or add an appropriate amount of water to dilute to a total volume of 24 mL for liquid products. Swirl to mix well.

(b) Add 10 mL 50% methanol to each sample. Swirl to mix well.

(c) Add 7.5 mL 2% metaphosphoric acid to each sample. Immediately swirl to mix well.

(d) Dilute the volumetric flasks to volume with laboratory water. Invert the flask to mix well.

(e) Transfer approximately 2 mL of each sample into a microcentrifuge tube. Centrifuge at 10 000 × *g* for 10 min.

(f) Filter the supernatant of each sample through syringe filter into an HPLC autosampler vial.

F. Instrumentation

(a) *Instrumental operating conditions*.—(*1*) *FD wavelength parameters*.—λ_{ex} = 495 nm, λ_{em} = 518 nm.

(*2*) *Run time*.—15 min.

(*3*) *Injection volume*.—20 µL.

(*4*) *Column temperature*.—20°C.

(*5*) *Mobile phase flow rate*.—For each injection run, the mobile phase flow rate starts at 0.4 mL/min and the column

switch valve position starts at 1→2. At 5 min, after biotin is eluted and detected by the FD, the valve position is switched to 1→6. The flow rate then ramps to 1.5 mL/min over 1 min and keeps until 12 min, when riboflavin is eluted and detected by the FD. Lastly, the flow rate decreases to 0.4 mL/min over 1 min before the valve position is switched back to 1→2. The flow rate keeps at 0.4 mL/min until 15 min.

(*6*) *Postcolumn pump flow rate*.—0.2 mL/min.

(b) *Instrument configuration with an optional column switch*.—The system should be configured as shown in Figure **2016.11A**.

(c) *System pressure*.—Column pump head pressure maximum at 600 bar or per column manufacturer's instructions; postcolumn reaction coil head pressure maximum at 40 bar or per manufacturer's instructions.

(d) *System equilibration*.—(*1*) Turn on the FD at least 1 h before start of analysis.

(*2*) Inject the most concentrated standard (approximately 100 ng/mL) onto the column and observe the response on the FD. If necessary, adjust the detector gain and sensitivity settings so that the standard curve is within the range of the detector. After the detector settings have been determined, inject the most concentrated standard three to four 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%, then 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. After 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.

(e) *Column and system maintenance*.—The column, postcolumn reaction coil, and system may be cleaned by using 50% methanol at an appropriate pressure, referring to **F(c)**.

G. Calculations

Quantification is obtained by using a seven-level external standard consisting of the following concentrations: 5, 10, 20, 40, 60, 80, and 100 ng/mL. The calibration of the standards is determined by using a polynomial regression curve (cubic-fit).

(a) Calculation of stock standard concentration:

$$SS = S_w \times P \div 500 \times D_1$$

where SS = the stock standard concentration (µg/mL); S_w = the standard weight (g); P = the purity of the standard (g/g); 500 = the volume of the stock solution (mL); and D_1 = the unit conversion factor: D_1 = 1 000 000 µg/g.

(b) Calculation of intermediate standard (IS) concentration:

IS = (volume of SS used) × (SS concentration) ÷ dilution volume

(c) Calculation of working standards concentration level 7 (WS7):

WS7 = (volume of IS used in mL) × (concentration of IS in µg/mL) ÷ (WS7 volume in mL) × 1000 ng/µg

(d) Calculation of working standards concentration level 1–6 (WS1–6):

WS1–6 = (volume of WS7 used in mL) × (concentration of WS7 in ng/mL) ÷ (WS1–6 volume in mL)

(e) Calculation of the biotin concentration in the injected product samples (C_i) is from its biotin peak area and the standard curve generated from the standards.

(f) Calculation of original product concentration (C_p) is, therefore, based on the dilution scheme used for the sample:

$$C_p = C_i \times D_0 \div ss \times D_1 \times D_2$$

where C_p = the original product concentration (µg/100g); C_i = the injected sample's biotin concentration, from standard curve (ng/mL); D_0 = the dilution of original product before filtration: D_0 = 50 mL; ss = the sample size (g); D_1 = the unit conversion (from per g to per 100 g sample and from ng to µg of biotin): D_1 = (100 g/100 g)(1 µg/1000 ng) = 1/10 g·µg/ng/100 g; and D_2 = the conversion from sample to reconstituted finished product: for powder, D_2 = 25 g powder ÷ (25 g powder + 200 g water) = 25 g ÷ 225 g = 1/9; for liquid (2× dilution by weight), D_2 = 2; and for ready-to-feed (RTF; as is), D_2 = 1.

Results and Discussion

Method Validation

This method has undergone a thorough single-laboratory validation (SLV) using AOAC INTERNATIONAL guidelines to probe its linearity, LOQ, specificity, accuracy, and ruggedness. The analytical range for SPIFAN biotin-fortified matrixes was found to be between 1.7 and 142 µg/g reconstituted final product or RTF.

Calibration fit.—During each analytical run, seven standards with biotin concentrations ranging from 5 to 100 ng/mL were injected before and after each sample set. Calibration curves were constructed from these standards using a polynomial regression curve (cubic-fit) and used to back-calculate the concentration of each working standard in order to calculate calibration error at each level. The method demonstrated good polynomial regression (cubic) fit, over a standard range of 5–100 ng/mL biotin, with r^2 >0.9990. The calibration errors for the lowest two levels (near the LOQ level) are around 25% and 10%; the calibration errors for the remaining levels were <8% (Table 1; Figure 2).

Suitable calibration curve range.—Due to the characteristics of the postcolumn protein binding reaction, saturated

Table 1. Representative calibration standard data

Level	Concentration, ng/mL	HPLC peak area	Amount, ng/mL	Error, %
1	5	0.2019	6.21	24
2	10	0.6600	9.00	10
3	20	2.2932	18.61	7
4	40	6.1743	41.03	3
5	60	9.2305	60.45	1
6	80	11.353	77.66	3
7	100	12.863	103.9	4

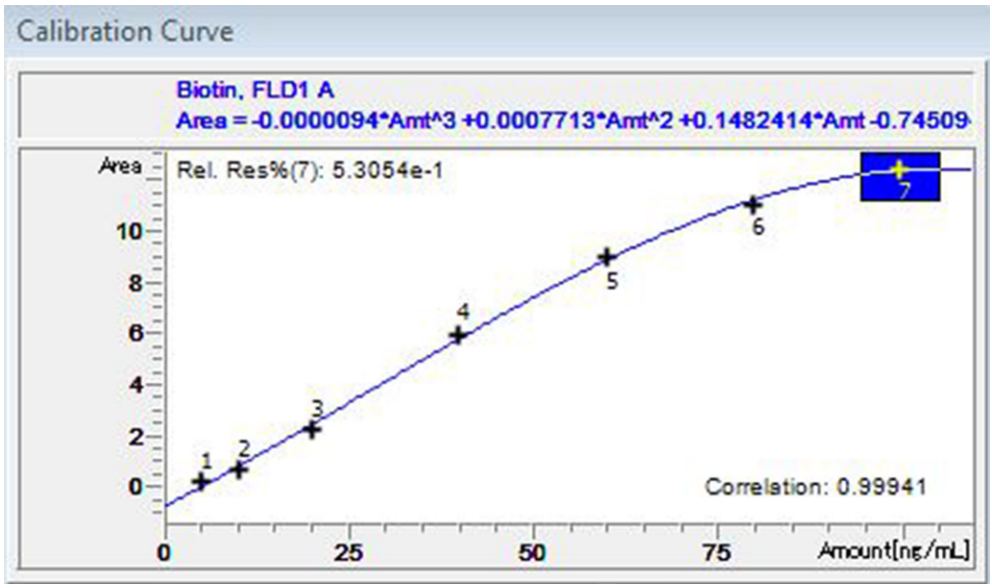


Figure 2. Representative calibration curve.

response occurs when biotin is injected at high concentration. To achieve accurate quantification, the method requires an appropriate sample size to ensure that the response (peak area) of the injected sample fits within the established calibration curve range. Based on the recovery data of Standard Reference Material (SRM) 1849a with different sample sizes, the suitable range of the calibration curve is 8.5–60 ng/mL for this method (Table 2). If the biotin concentration of the injected sample is >60 ng/mL, a further dilution with mobile phase is required.

Precision.—All fortified and unfortified matrixes were freshly prepared and analyzed in duplicate on 6 days. The SMPRs require RSD_r to be ≤6% for matrixes that contain >1 µg biotin/100 g reconstituted final product. The RSD_r obtained from the SLV ranged from 0.5 to 3.0% and met the requirements. The intermediate RSD_R was in the 1.3–4.5% range (Table 3).

Accuracy.—A total of 11 representative SPIFAN matrixes were spiked with biotin dissolved in 0.5% ethanol. Fortified matrixes were spiked at either 100% or 50% of the previously determined biotin level, and placebos were spiked at either 150% or 50% of the previously determined biotin level in their corresponding fortified matrixes. The spiked sample was either stored at room temperature for 2 h or stored refrigerated for 24 h to allow biotin to become incorporated into the sample matrix. The spiked samples were prepared and analyzed in duplicate on 3 days. In addition, SRM 1849a with different sample sizes was prepared and analyzed. The result was compared to the certificate of authenticity value.

The SMPRs require recovery to be 90–110% for matrixes that contain >1 µg biotin/100 g reconstituted final product. The requirements were met for 10 of 11 tested SPIFAN matrixes. The mean spike recovery data ranged between 95 and 111% (Table 4).

Recovery of SRM1849a met the requirement (Table 5).

LOQ.—Biotin LOD and LOQ values were determined experimentally by spiking a very low level of biotin into placebos. Blank mean and SD were obtained from eight injections.

$$\text{LOQ} = \text{blank mean} + 10\ \text{SDs}$$

The result was confirmed by the S/N method. A placebo was spiked with biotin at the estimated LOQ level and the peak S/N was 10.

The LOQ was estimated to be 0.8 µg/100 g reconstituted final product for powder, assuming a 4 g sample was diluted to 50 mL; and 1.5 µg/100 g for RTF, assuming a 20 g sample was diluted to 50 mL. The biotin levels in all the SPIFAN matrixes are above the LOQ of this method.

Table 2. Suitable range of sample concentration after dilution

SRM Product	Code	Reference value, mg/kg powder	Sample size, g	HPLC-injected sample concn, ng/mL	Measured value, mg/kg powder	Recovery versus CoA, %[a]	Calibration standard		Suitable range concn, ng/mL
							Level	Concn, ng/mL	
1849a	CLC-10b	1.99	0.1445	6.276	2.172	109	Blank	0	8.5–60
			0.1532	6.981	2.278	114	1	4.981	
			0.2006	8.454	2.107	106	2	9.962	
			1.0020	40.21	2.006	101	3	19.92	
			1.0006	41.05	2.051	103	4	39.85	
			1.5177	58.69	1.934	97.2	5	59.77	
			1.5082	57.60	1.910	96.0	6	79.70	
			2.0006	69.39	1.734	87.1	7	99.62	
			2.0003	69.28	1.732	87.0			

[a] CoA = Certificate of Analysis.

Table 3. Precision

Product	SPIFAN code	No. replicates[a]	Concn level, µg/100 g reconstituted	RSD_r, %	RSD_{iR}, %[b]
Child Formula Powder, placebo	00847RF00	6	Not detected	NA[c]	NA
Infant Elemental Powder, placebo	00796RF00	6	Not detected	NA	NA
Adult Nutritional RTF, High-Protein, placebo	00821RF00	6	Not detected	NA	NA
Adult Nutritional RTF, High-Fat, placebo	00820RF00	6	Not detected	NA	NA
Infant Formula RTF, Milk-Based, placebo	EV4H2Q	6	Not detected	NA	NA
SRM 1849a	CLC10-b	12	22.4[d]	1.5	2.6
Infant Formula Powder, Partially Hydrolyzed Milk-Based	410057652Z	12	4.07	0.5	1.6
Infant Formula Powder, Partially Hydrolyzed Soy-Based	410457651Z	12	4.43	0.5	1.3
Toddler Formula Powder, Milk-Based	4052755861	12	10.7	1.9	4.5
Infant Formula Powder, Milk-Based	4044755861	12	2.90	2.5	3.3
Adult Nutritional Powder, Low-Fat	00859RF00	12	31.9	0.7	2.8
Child Formula Powder	00866RF00	12	21.6	1.2	1.8
Infant Elemental Powder	00795RF00	12	10.7	1.9	4.5
Infant Formula Powder, FOS/GOS-Based[e]	50350017W1	12	1.66	2.6	4.1
Infant Formula Powder, Milk-Based	K16NTAV	12	5.11	1.2	3.0
Infant Formula Powder, Soy-Based	E10NWZC	12	5.15	3.0	4.0
Infant Formula RTF, Milk-Based	EV4H2R	12	3.86	1.7	4.1
Adult Nutritional RTF, High-Protein	00730RF00	12	56.8	1.0	3.4
Adult Nutritional RTF, High-Fat	00729RF00	12	76.2	0.6	3.1

[a] Duplicates on 3 or 6 days.

[b] RSD_{iR} = Intermediate RSD_R.

[c] NA = Not applicable.

[d] Reference value taken from the Certificate of Analysis of SRM 1849a.

[e] FOS/GOS = Fructo-oligosaccharide/galacto-oligosaccharide.

Table 4. Accuracy: spike recovery

				Spike recovery			
			Native level, µg/100 g reconstituted	Level 1 (~50%)		Level 2 (~150% for placebo, ~100% for fortified)	
Product	SPIFAN code	No. replicates		Avg., % (n = 3)	RSD, %	Avg., % (n = 3)	RSD, %
Child Formula Powder, placebo	00847RF00	6	Not detected	103	3.2	105	1.5
Adult Nutritional RTF, High-Protein, placebo	00821RF00	6	Not detected	102	1.6	103	1.5
Adult Nutritional RTF, High-Fat, placebo	00820RF00	6	Not detected	102	2.2	104	1.7
Infant Formula Powder, Partially Hydrolyzed Soy-Based	410457651Z	6	4.43	105	4.2	101	4.2
Adult Nutritional Powder, Low-Fat	00859RF00	6	31.9	111	2.9	102	1.8
Child Formula Powder	00866RF00	6	21.6	109	2.8	104	1.7
Infant Elemental Powder	00795RF00	6	10.7	106	2.8	95.1	3.0
Infant Formula Powder, FOS/GOS-Based[a]	50350017W1	6	1.66	105	6.4	99.9	7.2
Infant Formula Powder, Milk-Based	K16NTAV	6	5.11	104	6.1	103	4.6
Adult Nutritional RTF, High-Protein	00730RF00	6	56.8	109	1.6	102	0.8
Adult Nutritional RTF, High-Fat	00729RF00	6	76.2	109	2.4	101	0.7

[a] FOS/GOS = Fructo-oligosaccharide/galacto-oligosaccharide.

Specificity.—Chromatograms from all of the SPIFAN matrixes helped to establish the specificity of this method for biotin. *See* Table 6 for representative chromatograms of tested SPIFAN matrixes.

Ruggedness.—Several parameters were varied during validation to establish method ruggedness. Samples were prepared by three analysts and analyzed with C18 columns from four different lots. Fresh mobile phase, postcolumn reagents, intermediate standards, and working standards were made daily and used during validation.

Conclusions

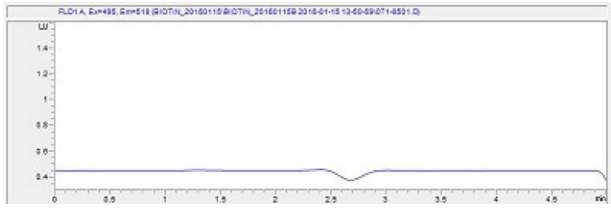
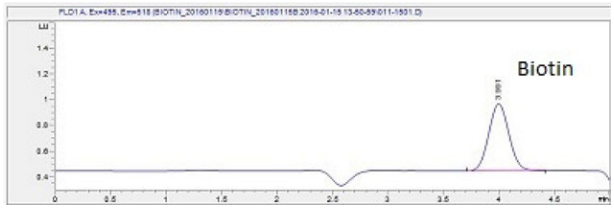
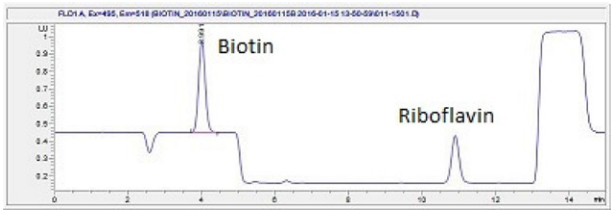
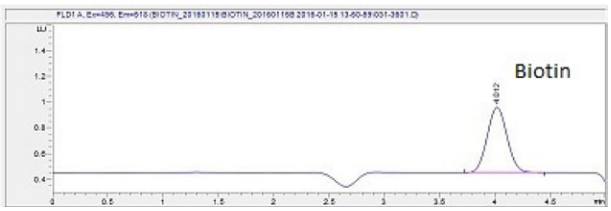
The data presented in this paper were submitted to the AOAC Expert Review Panel (ERP) for review at the AOAC Mid-Year Meeting held on March 16, 2016. The ERP determined that the data presented here were in accordance with SMPR 2014.005 (5) approved by SPIFAN, and the method was granted First Action status.

Table 5. Accuracy: recovery of SRM 1849a

SRM product	Code	Reference value, mg/kg powder	Measured value, mg/kg powder	Recovery versus CoA, %[a]
1849a	CLC-10b	1.99	1.969	99
			2.067	104
			2.081	105
			2.023	102
			2.047	103
			1.971	99
			2.013	101
			1.987	100
			1.995	100

[a] CoA = Certificate of Analysis.

Table 6. Representative chromatograms of tested SPIFAN matrices

Matrix	SPIFAN Code	Chromatogram
Child Formula Powder, placebo	00847RF00	
		
		Full-time range to include the riboflavin peak (*see* below)
SRM 1849a	CLC10-b	
Child Formula Powder	00866RF00	

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Lutein and β-Carotene in Infant Formula and Adult Nutritionals by Ultra-High-Performance Liquid Chromatography: Single-Laboratory Validation, First Action 2016.13

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An ultra-HPLC method for the determination of lutein and β-carotene in infant formula and adult nutritionals was validated using both unfortified and fortified samples provided by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN). All experiments showed separation of all-*trans*-lutein and β-carotene from their major *cis* isomers, apocarotenal, α-carotene, lycopene, and zeaxanthin. Samples spiked with all-*trans*-lutein and β-carotene showed no isomerization during sample preparation. Linearity of the calibration solutions correlated to approximately 0.8–45 µg/100 g (reconstituted basis) for samples prepared for the lowest sample concentrations. With dilutions specified in the method, the range can be extended to approximately 2250 µg/100 g. The LOD for both lutein and β-carotene was 0.08 µg/100 g, and the LOQ for both was 0.27 µg/100 g. For all measurements in the range of 1–100 µg/100 g, repeatability RSD was ≤5.8% for lutein and ≤5.1% for β-carotene. For measurements >100 µg/100 g, repeatability RSD was ≤1.1% for lutein and ≤1.7% for β-carotene. Accuracy was determined by recovery from spiked samples and ranged from 92.3 to 105.5% for lutein and from 100.1 to 107.5% for β-carotene. The data provided show that the method meets the criteria specified in the *Standard Method Performance Requirements* for carotenoids (SMPR 2014.014).

The carotenoids present in human milk include α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene (1–3). Of these, lutein and β-carotene are most commonly added to infant formula and adult nutritionals. Whereas β-carotene has provitamin A activity (4), lutein may play a role in vision and cognitive function (5). Both lutein and β-carotene can occur as all-*trans* and *cis* isomers, and there is interest in separating these because of differences in absorption and biological activity. In addition to having twice the vitamin A activity of *cis* isomers, all-*trans*-β-carotene is preferentially absorbed over 9-*cis*-β-carotene (6). All-*trans*-lutein is the most common isomer found in human retinas (7) and infant brains (8).

Because there were no official methods for the determination of lutein or β-carotene in infant formula and adult nutritionals, the current method was developed based on existing extraction and chromatographic procedures from various carotenoid methods. The saponification procedure was adapted from Granado et al. (9), the extraction solvents from Craft (10), the use of apocarotenal as an internal standard from Marx et al. (11), and the use of 10 mM α-tocopherol as an antioxidant from Scita (12). Chromatographic separation of lutein and β-carotene isomers with C30 columns and a methanol–methyl tert-butyl ether (MTBE) mobile phase has been demonstrated in several reports (11, 13–16), and the current method adapted these procedures for optimum resolution, sensitivity, and run time. Calculations for standard concentrations were based on purity from Müller et al. (17) using extinction coefficients from Craft and Soares (18). Response factors for *cis* isomers of β-carotene relative to the all-*trans* form were taken from Schierle et al. (13) and align with AOAC *Official Method*^{SM} **2005.07** (19) and the United States Pharmacopeia (USP) monograph for β-carotene (20).

AOAC Official Method 2016.13
Lutein and β-Carotene in Infant Formula and Adult Nutritionals
Reversed-Phase Ultra-High-Performance Liquid Chromatography
First Action 2016

(Applicable to the determination of all-*trans*-lutein, *cis* isomers of lutein, all-*trans*-β-carotene, and *cis* isomers of β-carotene in infant formula and adult nutritionals from 1 to 1300 µg/100 g reconstituted basis. Materials tested must not contain measurable levels of β-apo-8′-carotenal.)

Caution: Tetrahydrofuran (THF) can form peroxides, and only THF stabilized with butylated hydroxytoluene (BHT) should be used. Refer to Material Safety Data Sheets when using any reagent, and use appropriate personal protective equipment when performing analyses.

Note: Throughout this method, estimated sample concentrations for standard and sample preparations are stated per 100 g on a reconstituted basis [as is for ready-to-feed (RTF)

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This method was approved by the AOAC Expert Review Panel for SPIFAN Nutrient Methods as First Action.
The Expert Review Panel for SPIFAN Nutrient Methods 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: gregory.hostetler@perrigo.com
DOI: 10.5740/jaoacint.16-0386

liquids, 25 g sample plus 200 g water for powder samples, or diluted 1:1 by weight for liquid concentrates] in accordance with *Standard Method Performance Requirement* (SMPR®) 2014.014 (21). The test sample concentrations calculated in **H(f, g)** are expressed per 100 g on an as is basis for all samples, allowing each laboratory to apply appropriate reconstitution factors.

A. Principle

Test samples (reconstituted powders, liquid RTFs, and liquid concentrates) are spiked with an internal standard and saponified with potassium hydroxide. Samples are then extracted with MTBE and THF, followed by hexane. The supernatants from the liquid–liquid extraction are dried under nitrogen and reconstituted in 2-propanol (isopropyl alcohol; IPA). Separation is performed by reversed-phase chromatography on a C30 column. All-*trans*-lutein and β-carotene are separated from their major *cis* isomers, as well as from zeaxanthin, α-carotene, and lycopene.

B. Apparatus

Note: Carotenoids are light sensitive, and all work should be done under protection from UV light.

(a) *Ultra-HPLC (UHPLC) system*.—Consisting of binary pump, autosampler, thermostatted column compartment, UV-Vis detector with 60 mm flow cell, and data acquisition software.

(b) *Analytical column*.—C30 carotenoid column, 3 µm, 2.0 × 250 mm (Part No. CT99S03-2502WT; YMC, Kyoto, Japan).

(c) *Guard column*.—C30 guard column, 3 µm, 2.1 × 10 mm (Part No. CT99S03-01Q1GC; YMC).

(d) *Guard cartridge holder*.—Part No. XPGCH-Q1 (YMC).

(e) *Spectrophotometer*.—Wavelength range of 200–700 nm, with 1 cm quartz cells.

(f) *Top-loading balance*.—Capable of weighing to 0.1 g.

(g) *Analytical balance*.—Capable of weighing to 0.01 mg.

(h) *Ultrasonic water bath*.—40 kHz.

(i) *Reciprocating shaker*.—Capable of 200 rpm.

(j) *Evaporator*.—With pure nitrogen supply.

(k) *Laboratory centrifuge*.—With adapters for 50 mL centrifuge tubes.

(l) *Centrifuge tubes*.—50 mL, polypropylene.

(m) *Syringes*.—1 mL, disposable.

(n) *Syringe filters*.—0.2 µm, PTFE.

(o) *Class A volumetric flasks*.—Various sizes; clear and amber.

(p) *Scintillation vials*.—12 mL, amber.

(q) *HPLC vials*.—Amber, with 300 µL inserts.

(r) *Class A volumetric pipets*.—Various sizes.

C. Reagents

Note: Reagent volumes may be scaled up or down provided good laboratory practices are followed.

(a) *Laboratory water*.—>18 megohm-cm.

(b) *Methanol (MeOH)*.—HPLC grade.

(c) *MTBE*.—HPLC grade.

(d) *n-Hexane*.—HPLC grade.

(e) *Potassium hydroxide (KOH)*.—Pellets, ACS grade.

(f) *Reagent alcohol (ROH)*.—Denatured ethanol, HPLC grade.

(g) *α-Tocopherol (vitamin E)*.—95%.

(h) *Pyrogallic acid (pyrogallol)*.—ACS grade.

(i) *IPA*.—HPLC grade.

(j) *THF*.—99.9%, stabilized with BHT.

(k) *Ammonium acetate*.—HPLC grade, 98%.

(l) *Potassium hydroxide solution, 50% (w/w)*.—Add 50 mL water to a 250 mL beaker. Weigh 50 g KOH and slowly transfer to the beaker under constant stirring. When dissolved and cooled, transfer to a media bottle and store at room temperature for up to 6 months.

(m) *MTBE/vitamin E*.—Dissolve 2.2 g α-tocopherol in 500 mL MTBE. Store in the refrigerator for up to 1 month.

(n) *Pyrogallol solution (0.2 M pyrogallic acid in ethanol)*.—Dissolve 6.3 g pyrogallic acid in 250 mL ROH. Store in the refrigerator for up to 1 month. Solution should be clear at room temperature; discard if colored.

(o) *Extraction solution [10 mM vitamin E in MTBE–THF (1 + 1)]*.—Dissolve 2.2 g α-tocopherol in 250 mL MTBE and 250 mL THF. Store in the refrigerator for up to 1 month.

(p) *Sample solvent (10 mM vitamin E in IPA)*.—Dissolve 4.4 g α-tocopherol in 1000 mL IPA. Store in the refrigerator for up to 1 month.

(q) *Mobile phase for LC system*.—*(1) Phase A*.—20 mM ammonium acetate in methanol–water (98 + 2). Combine 980 mL MeOH, 20 mL water, and 1.54 g ammonium acetate and mix to dissolve. Store at room temperature for up to 1 month.

(2) Phase B.—MTBE.

D. Standards

(a) *Lutein*.—ChromaDex (Part No. ASB-00012453; Irvine, CA), or equivalent.

(b) *β-Carotene*.—USP (Part No. 1065480; Rockville, MD), or equivalent.

(c) *Apocarotenal (β-apo-8'-carotenal)*.—USP Part No. 1040854, or equivalent.

(d) *Lutein containing approximately 10% zeaxanthin*.—USP Part No. 1370804.

(e) *β-Carotene system suitability reference standard*.—USP Part No. 1065491.

E. Standards Preparation

Standard solution preparation is summarized in Table **2016.13A** and detailed below.

(a) *Carotenoid stock solutions (20 000 µg/100 mL)*.—Weigh (to 0.01 mg) approximately 10 mg each of the lutein, **D(a)**; β-carotene, **D(b)**; and apocarotenal, **D(c)** reference standards into separate 50 mL volumetric flasks. Add approximately 40 mL MTBE/vitamin E, **C(m)**, to each, sonicate for 2 to 3 min, and dilute to volume with MTBE/vitamin E. Store stock solutions at −20°C for up to 6 months and check their purity each time new standard solutions are made from them.

(b) *Standard measuring solutions for UV-Vis spectroscopy potency check (200 µg/100 mL)*.—Transfer 1.0 mL aliquots of lutein and β-carotene standard stock solutions, **E(a)**, to

Table 2016.13A. Composition and nominal concentrations of carotenoid standard solutions

	Stock solution (20000 µg/100 mL) in MTBE/vitamin E		UV-Vis solution (200 µg/100 mL) in MTBE		Working solution (200 µg/100 mL) in sample solvent		Intermediate solution in sample solvent		
Analyte	Standard, mg	Total volume, mL	Stock solution, mL	Total volume, mL	Stock solution, mL	Total volume, mL	Stock solution, mL	Total volume, mL	Concn, µg/100 mL
Lutein	10	50	1.0	100	0.1	10	2.0	100	400
β-Carotene	10	50	1.0	100	0.1	10	2.0		400
Apocarotenal	10	50	—	—	1.0	100	3.0	50	1200

separate 100 mL volumetric flasks and dilute each to volume with MTBE. Prepare fresh when needed.

(c) *Individual carotenoid working solutions for chromatographic purity check (200 µg/100 mL).*—(*1*) *For lutein and β-carotene*.—Transfer 100 µL aliquots of each standard stock solution, **E(a)**, to separate 10 mL volumetric flasks and dilute each to volume with sample solvent. Prepare fresh when needed.

(*2*) *For apocarotenal*.—Transfer 1.0 mL standard stock solution, **E(a)**, to a 100 mL volumetric flask and dilute to volume with sample solvent. Store at −20°C for up to 1 month and use for internal standard, **E(i)**.

(d) *Apocarotenal intermediate solution (1200 µg/100 mL).*—Transfer 3.0 mL apocarotenal stock solution, **E(a)**, to a 50 mL volumetric flask and dilute to volume with sample solvent. Store at −20°C for up to 1 month.

(e) *Mixed carotenoid intermediate solution (400 µg/100 mL).*—Combine 2.0 mL each of lutein and β-carotene standard stock solutions, **E(a)**, in a 100 mL volumetric flask and dilute to volume with sample solvent. Store at −20°C for up to 1 month.

(f) *Calibration solutions.*—Transfer apocarotenal intermediate solution, **E(d)**, and mixed carotenoid intermediate solution, **E(e)**, to volumetric flasks according to Table **2016.13B** and dilute to volume with sample solvent. Store at −20°C for up to 1 month.

(g) *β-Carotene system suitability solution.*—Transfer 20 mg β-carotene system suitability reference standard, **D(e)**, to a 50 mL volumetric flask. Add 1 mL water and 4 mL THF and sonicate for 5 min. Dilute to volume with IPA and sonicate for 5 min. Cool to room temperature, filter the cloudy suspension through 0.2 µm PTFE, and dilute the clear filtrate 1:4 with IPA. Store in the refrigerator for up to 3 months.

(h) *Lutein system suitability solution.*—Transfer 10 mg USP lutein, **D(d)**, to a 100 mL volumetric flask, dilute to volume with ROH, and mix. Transfer 1 mL to a 50 mL volumetric flask. Add approximately 35 mL IPA, stopper loosely, and heat in a water bath at 80°C for 2 hours. Cool to room temperature, add 250 µL apocarotenal stock solution, **E(a)**, and dilute to volume with IPA. Store in the refrigerator for up to 3 months.

(i) *Internal standard solution (ISTD).*—Prepare immediately before use.—(*1*) *For infant formula and samples with low carotenoid concentrations (up to 100 µg individual carotenoid per 100 g)*.—Transfer 4.0 mL apocarotenal working solution, **E(c)**, to a 50 mL volumetric flask and dilute to volume with pyrogallol solution, **C(n)**. This is enough solution for nine samples.

(*2*) *For samples with individual carotenoid concentrations >100 µg/100 g*.—Transfer 4.0 mL apocarotenal intermediate solution, **E(d)**, to a 50 mL volumetric flask and dilute to volume with pyrogallol solution, **C(n)**.

F. Sample Preparation

Note on range: Although this method can quantify carotenoids in the range of 1–1300 µg/100 g, it is recommended to quantify only a 100-fold difference with a single preparation. For example, the range of 1–100 µg/100 g works well for infant formula, but the range of 15–1500 µg/100 g would work best for samples with the highest carotenoid concentrations.

(a) Prepare up to nine samples at a time.

(b) *Weights*.—Weigh all samples (powders and liquids) to 0.1 mg.—(*1*) *Powders*.—Weigh approximately 625 mg powder sample into a 50 mL centrifuge tube. Add 5 mL water, cap, and vortex-mix until dissolved. Let sit for up to 15 min at room temperature.

Note: For nonhomogeneous powder samples, first dissolve 25 g powder sample with 200 mL water (record weights of both powder sample and water) and then transfer approximately 5 g reconstituted sample into a 50 mL centrifuge tube.

(*2*) *Liquid RTF with individual carotenoid concentrations ≤200 µg/100 g*.—Shake bottle or can on a reciprocating shaker 10 min before opening. Transfer approximately 5 g sample into a 50 mL centrifuge tube.

Table 2016.13B. Composition and nominal concentrations of carotenoid calibration solutions

Calibration solution	Apocarotenal intermediate solution, mL	Mixed carotenoid intermediate solution, mL	Total volume, mL	Lutein concn, µg/100 mL	Apocarotenal concn, µg/100 mL	β-Carotene concn, µg/100 mL
1	2.0	15.0	25	240	96	240
2	2.0	8.0	25	128	96	128
3	2.0	5.0	25	80	96	80
4	2.0	2.0	25	32	96	32
5	8.0	1.0	100	4	96	4

Table 2016.13C. Chromatographic conditions

Parameter	Condition	
Analytical column	YMC C30 3 µm, 250 × 2.0 mm	
Guard column	YMC C30 3 µm, 10 × 2.0 mm	
Column temperature	30°C	
Mobile phases	A: 20 mM ammonium acetate in MeOH–water 98 + 2; B: MTBE	
	Time, min	Mobile phase B, %
Gradient	0	5
	1	8
	8	15
	25	100
	25.5	5
	32	5
Flow rate	0.25 mL/min	
Backpressure	~185 bar	
Injection volume	5 µL	
UV/Vis detection	450 nm, ref = off	

(*3*) *RTF sample with individual carotenoid concentrations >200 µg/100 g*.—Shake bottle or can on a reciprocating shaker 10 min before opening. Transfer approximately 2 g sample into a 50 mL centrifuge tube. Add 3 mL water, cap, and vortex-mix 10 s. Let sit for up to 15 min at room temperature.

(*4*) *Infant formula concentrate*.—Shake bottle or can on a reciprocating shaker 10 min before opening. Transfer approximately 2.5 g sample into a 50 mL centrifuge tube. Add 2.5 mL water, cap, and vortex-mix 10 s. Let sit for up to 15 min at room temperature.

(c) Pipet a 5.0 mL aliquot of the appropriate ISTD from **E(i)** to each tube.

(d) Add 1.5 mL KOH solution, **C(l)**, to each tube with a repeater pipet.

(e) Shake on reciprocating shaker for 5 min.

(f) Add 8 mL extraction solution, **C(o)**, to each tube with a repeater pipet.

(g) Shake for 10 min.

(h) With a repeater pipet or dispenser, add 10 mL water and 10 mL hexane to each tube.

(i) Shake for 1 min.

(j) Centrifuge at 1000 rpm (or equivalent to 200 × *g*) for 5 min.

(k) *Supernatant volume*.—Transfer a portion of the supernatant to a 12 mL scintillation vial.—(*1*) *For samples with individual carotenoid concentrations ≤50 µg/100 g*.—Use 10 mL supernatant.

(*2*) *For samples with individual carotenoid concentrations >50 µg/100 g*.—Use 3 mL supernatant.

(l) Dry under nitrogen at ≤40°C.

(m) *Reconstitution volume*.—Reconstitute dried extract in sample solvent and vortex-mix.—(*1*) *For samples with individual carotenoid concentrations ≤100 µg/100 g*.—Add 0.5 mL.

(*2*) *For samples with individual carotenoid concentrations of 100–500 µg/100 g*.—Add 1 mL.

(*3*) *For samples with individual carotenoid concentrations of 500–1000 µg/100 g*.—Add 2 mL.

(*4*) *For samples with individual carotenoid concentrations of 1000–1500 µg/100 g*.—Add 3 mL.

(n) Filter through 0.2 µm PTFE syringe filter before injection.

G. Chromatography

(a) *Chromatographic conditions*.—Set up the UHPLC system according to the specifications in Table **2016.13C**. Follow the manufacturer's instructions for column installation, cleaning, and storage.

(b) *System suitability checks*.—(*1*) *Resolution between lutein cis and trans isomers*.—Inject the lutein system suitability solution, **E(h)**, and determine the resolution between the two major *cis* isomers and all-*trans*-lutein. Resolution should be ≥1.4 between 13-*cis*- and 13′-*cis*-lutein and ≥2.2 between 13′-*cis*- and all-*trans*-lutein using the half-width method. *See* Figure **2016.13A**.

(*2*) *Resolution between all-trans-lutein, zeaxanthin, and apocarotenal*.—From the chromatogram of the lutein system suitability solution, **E(h)**, determine the resolution between all-*trans*-lutein, zeaxanthin, and apocarotenal. Resolution should be

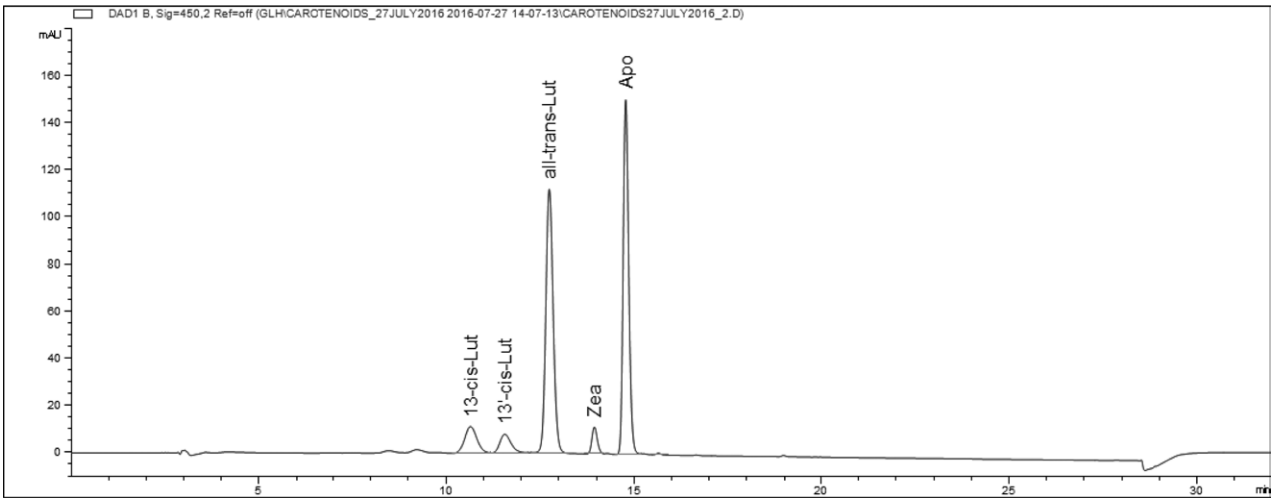

Figure 2016.13A. Chromatogram of lutein system suitability solution, E(h). Lut = lutein, Zea = zeaxanthin, and Apo = apocarotenal.

≥3.7 between all-*trans*-lutein and zeaxanthin and ≥2.5 between zeaxanthin and apocarotenal. *See* Figure **2016.13A**.

(*3*) *Resolution between β-carotene cis and trans isomers and α-carotene*.—Inject the β-carotene system suitability solution, **E(g)**, and determine the resolution between the two major *cis* isomers of β-carotene, all-*trans*-β-carotene, and α-carotene. Resolution should be ≥1.7 between 13-*cis*-β-carotene and *cis*/*trans*-α-carotene and ≥2.6 between all-*trans*-β-carotene and 9-*cis*-β-carotene. *See* Figure **2016.13B**.

(*4*) Inject the calibration solutions before and after each set of sample injections (up to 12 samples in each set). Calculate the slope relative to the internal standard as shown in **H(d)**. The coefficient of determination (R^2) for each curve should be >0.995. The slopes from the two curves should not differ by more than 2.0%.

(*5*) Representative sample chromatograms are shown in Figures **2016.13C-E**.

H. Calculations

(a) Determine the purity of lutein and β-carotene standards by first determining the spectrophotometric purity and then the chromatographic purity of each. The overall purity is calculated as the product of the two measured purities.—(*1*) *Spectrophotometric purity*.—Measure each standard measuring solution, **E(b)**, against an MTBE blank at its absorbance maximum (444 nm for lutein and 450 nm for β-carotene). Calculate the spectrophotometric purity (SP) of each reference standard as the observed absorbance over the expected absorbance:

$$SP = \left(Abs_{MS} \times 50000\right) \Big/ \left(E_{1\%,1cm} \times W\right)$$

where Abs_{MS} = the absorbance of the standard measuring solution; 50 000 = the dilution factor; $E_{1\%,1cm}$ = the extinction

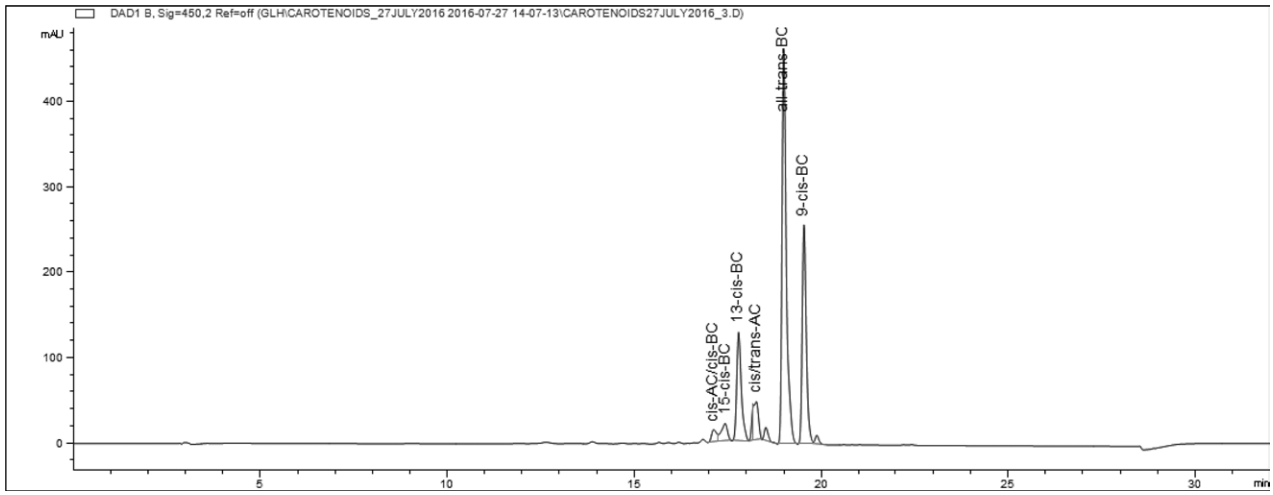

Figure 2016.13B. Chromatogram of β-carotene system suitability solution, E(g). AC = α-carotene and BC = β-carotene.

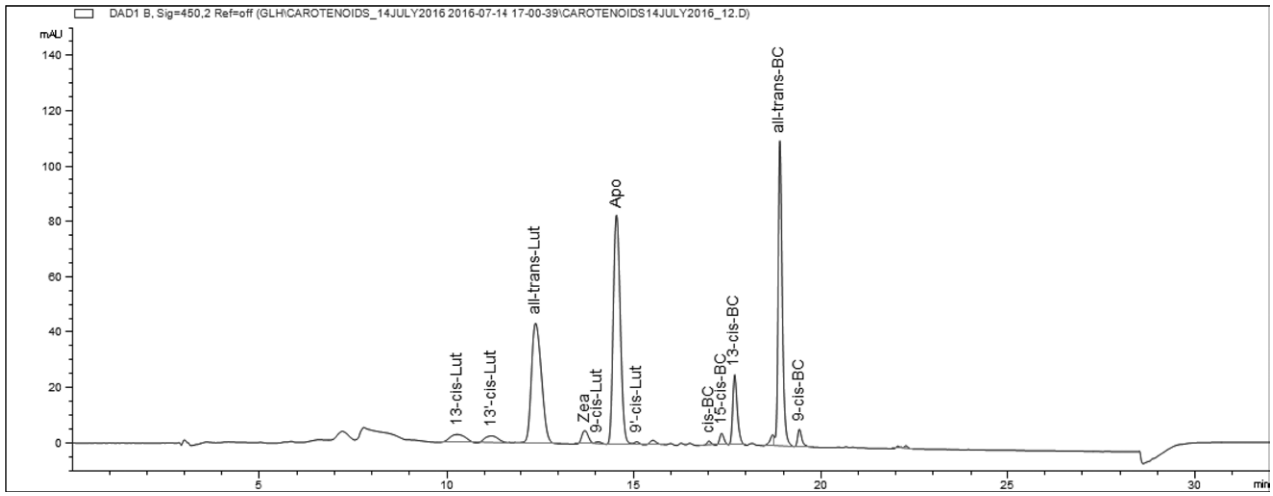

Figure 2016.13C. Chromatogram of a milk-based infant formula sample. Lut = lutein, Zea = zeaxanthin, Apo = apocarotenal, and BC = β-carotene.

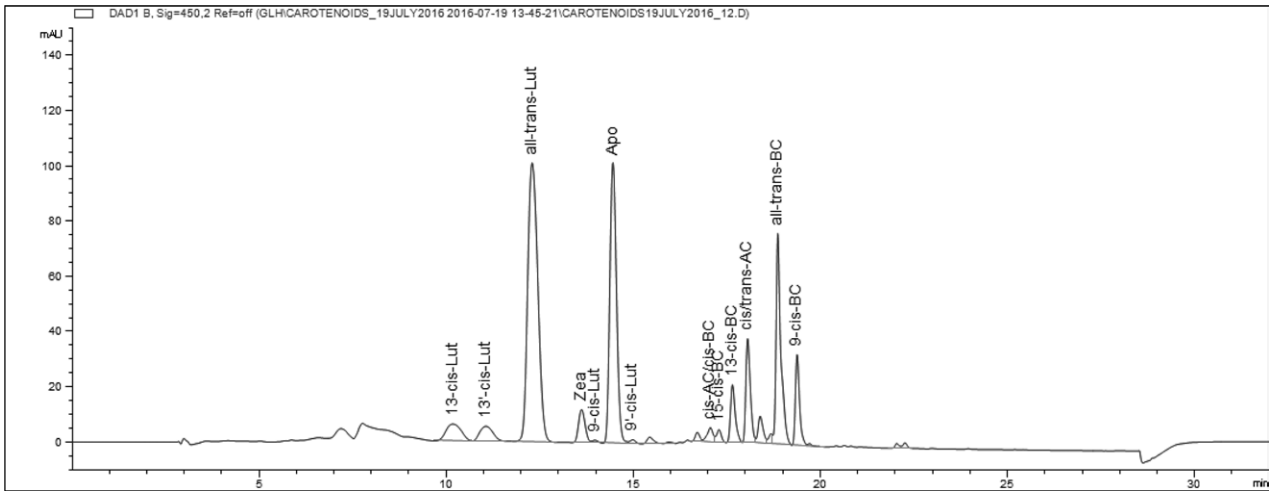

Figure 2016.13D. Chromatogram of a toddler formula sample. Lut = lutein, Zea = zeaxanthin, Apo = apocarotenal, AC = α-carotene, and BC = β-carotene.

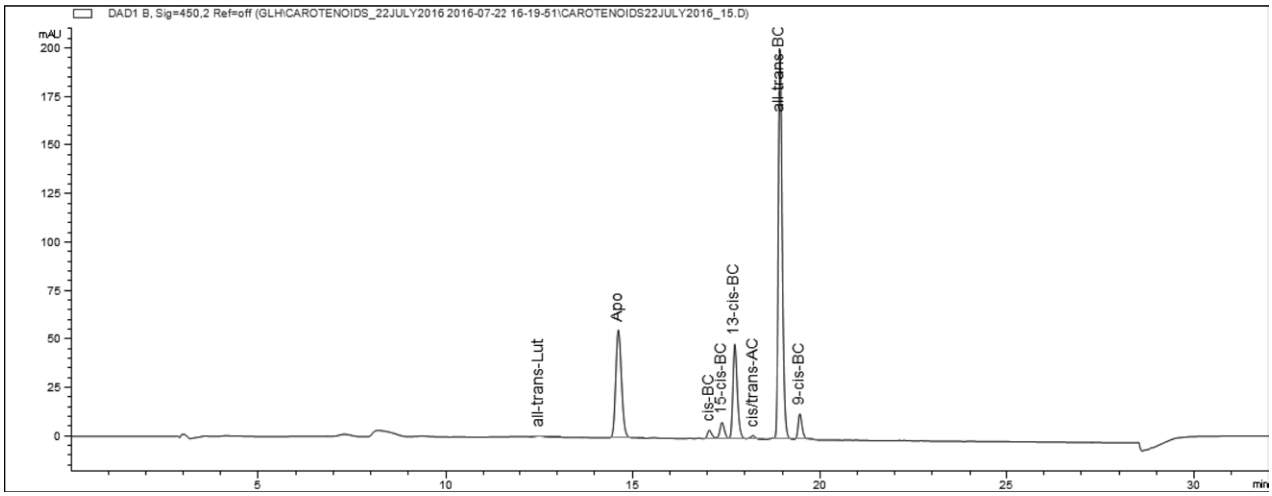

Figure 2016.13E. Chromatogram of an RTF adult nutritional sample. Lut = lutein, Apo = apocarotenal, AC = α-carotene, and BC = β-carotene.

coefficient (18; lutein in MTBE, 2589 at 444 nm; β-carotene in MTBE, 2588 at 450 nm); and W = the weight (mg) of reference standard. Spectrophotometric purity is typically greater than 0.90 (i.e., 90%).

(*2*) *Chromatographic purity.*—Inject standard working solutions, **E(c)**, at least three times. The chromatographic purity (CP) is calculated as

$$\mathrm{CP} = \left(\text{area of the all-}\textit{trans}\text{-carotenoid peak}\right) \Big/ \left(\text{sum of areas of all relevant peaks}\right)$$

Relevant peaks include all peaks in the HPLC chromatogram, with the exception of solvent peaks. Chromatographic purity is typically greater than 0.95 (i.e., 95%).

(*3*) *Reference standard purity.*—Calculate the purity (P) of each reference standard:

$$\mathrm{P} = \mathrm{SP} \times \mathrm{CP} \times 100$$

where SP = the spectrophotometric purity; CP = the chromatographic purity; and 100 = the factor for converting decimal to percent.

(b) Calculate the concentration (μg/100 mL) of each carotenoid analyte (e.g., C_{Lut} for lutein) in the all-*trans* form in each calibration solution, **E(f)**:

$$C_{Lut} = W_{Lut} \times 2 \times \left(P_{Lut}/100\right) \times 1000 \times (2/100) \times \left(V_{MC}/V_{Total}\right)$$

where W_{Lut} = the weight (mg) of lutein used to make the stock solution; 2 = the conversion of 50 mL to 100 mL; P_{Lut} = the reference standard purity of all-*trans*-lutein calculated in **H(a)**(*3*) above; 100 = the conversion from percent to decimal; 1000 = the conversion of milligrams to micrograms; (2/100) = the dilution of stock solution to mixed carotenoid intermediate solution; V_{MC} = the volume of mixed carotenoid intermediate solution, **E(e)**, used; and V_{Total} = the dilution volume.

(c) Calculate the concentration (μg/100 mL) of the apocarotenal internal standard (C_A) in each calibration solution, **E(f)**:

$$C_A = W_A \times 2 \times CP_A \times 1000 \times (3/50) \times \left(V_{AI}/V_{Total}\right)$$

where W_A = the weight (mg) of apocarotenal used to make the stock solution; 2 = the conversion of 50 mL to 100 mL; CP_A = the chromatographic purity of apocarotenal calculated in **H(a)**(*2*)

above; 1000 = the conversion of milligrams to micrograms; (3/50) = the dilution of stock solution to apocarotenal intermediate solution; V_{AI} = the volume of apocarotenal intermediate solution, **E(d)**, used; and V_{Total} = the dilution volume.

(d) For each calibration solution in **E(f),** calculate (*1*) the peak area ratio for each analyte: (peak area of all-*trans* lutein or β-carotene)/(peak area of internal standard); and (*2*) the concentration ratio: (concentration of all-*trans* lutein or β-carotene)/(concentration of internal standard). Build a five-point calibration curve with internal standard by plotting peak area ratios against concentration ratios, with relative concentration on the *x*-axis.

The accuracy on calibration points should be 100 ± 10%, and the coefficient of determination (R^2) should be greater than 0.995.

The calibration and calculation may be achieved through data processing within the instrument software or off-line.

(e) Calculate the mass (μg) of apocarotenal (M_A) added to the test samples:

$$M_A = \left(C_A \times V_A\right) \times \left(4/50\right)$$

where C_A = the concentration (μg/100 mL) of apocarotenal in the intermediate or working solution used in the ISTD; V_A = the volume (mL) of ISTD added to each sample; 4 = the volume (mL) of apocarotenal intermediate or working solution used in the ISTD; and 50 = the total volume (mL) of ISTD made.

(f) Calculate the contents of all-*trans*-lutein, *cis* isomers of lutein, and total lutein in the test samples. For peak identification, refer to relative retention times of peaks in Figures **2016.13A**, **2016.13C**, and **2016.13D**.

$$Lut_{trans} = \left(M_A/M_S\right) \times \left(\left[A_{Lut}/A_A\right] - I_{Lut}\right) \times \left(100/RF_{Lut}\right)$$

where Lut_{trans} = the concentration (μg/100 g) of all-*trans*-lutein in the sample; M_A = the mass (μg) of apocarotenal added to the test sample; M_S = the sample weight (g); A_{Lut} = the peak area (AU) of all-*trans*-lutein in the sample chromatogram; A_A = the peak area (AU) of apocarotenal in the sample chromatogram; I_{Lut} = the *y*-intercept of the calibration curve for all-*trans*-lutein; and RF_{Lut} = the slope of the calibration curve for all-*trans*-lutein.

$$Lut_{cis} = \left(M_A/M_S\right) \times \left(\left[\left(A_{13cisLut} + A_{13'cisLut} + A_{9cisLut} + A_{9'cisLut}\right)/A_A\right] - I_{Lut}\right) \times \left(100/RF_{Lut}\right)$$

where Lut_{cis} = the concentration (μg/100 g) of *cis* isomers of lutein in the sample; M_A = the mass (μg) of apocarotenal added to the test sample; M_S = the sample weight (g); $A_{13cisLut}$ = the peak area (AU) of 13-*cis*-lutein in the sample chromatogram; $A_{13'cisLut}$ = the peak area (AU) of 13′-*cis*-lutein in the sample chromatogram; $A_{9cisLut}$ = the peak area (AU) of 9-*cis*-lutein in the sample chromatogram; $A_{9'cisLut}$ = the peak area (AU) of 9′-*cis*-lutein in the sample chromatogram; A_A = the peak area (AU) of apocarotenal in the sample chromatogram; I_{Lut} = the *y*-intercept of the calibration curve for all-*trans*-lutein; and RF_{Lut} = the slope of the calibration curve for all-*trans*-lutein.

$$Lut_{Total} = Lut_{trans} + Lut_{cis}$$

(g) Calculate the contents of all-*trans*-β-carotene, *cis* isomers of β-carotene, and total β-carotene in the test samples. For peak identification, refer to relative retention times of peaks in Figures **2016.13B-D**.

$$BC_{trans} = \left(M_A/M_S\right) \times \left(\left[A_{BC}/A_A\right] - I_{BC}\right) \times \left(100/RF_{BC}\right)$$

where BC_{trans} = the concentration (μg/100 g) of all-*trans*-β-carotene in the sample; M_A = the mass (μg) of apocarotenal added to the test sample; M_S = the sample weight (g); A_{BC} = the peak area (AU) of all-*trans*-β-carotene in the sample chromatogram; A_A = the peak area (AU) of apocarotenal in the sample chromatogram; I_{BC} = the *y*-intercept of the calibration curve for all-*trans*-β-carotene; and RF_{BC} = the slope of the calibration curve for all-*trans*-β-carotene.

$$BC_{cis} = \left(M_A/M_S\right) \times \left(\left[\left(\left[A_{15cisBC} \times 1.4\right] + \left[A_{13cisBC} \times 1.2\right] + A_{9cisBC} + A_{XcisBC}\right)/A_A\right] - I_{BC}\right) \times \left(100/RF_{BC}\right)$$

where BC_{cis} = the concentration (μg/100 g) of *cis* isomers of β-carotene in the sample; M_A = the mass (μg) of apocarotenal added to the test sample; M_S = the sample weight (g); $A_{15cisBC}$ = the peak area (AU) of 15-*cis*-β-carotene in the sample chromatogram; $A_{13cisBC}$ = the peak area (AU) of 13-*cis*-β-carotene in the sample chromatogram; A_{9cisBC} = the peak area (AU) of 9-*cis*-β-carotene in the sample chromatogram; A_{XcisBC} = the peak area (AU) of unidentified *cis* isomers of β-carotene in the sample chromatogram; A_A = the peak area (AU) of apocarotenal in the sample chromatogram; I_{BC} = the *y*-intercept of the calibration curve for all-*trans*-β-carotene; and RF_{BC} = the slope of the calibration curve for all-*trans*-β-carotene.

$$BC_{Total} = BC_{trans} + BC_{cis}$$

Validation

Selectivity

SMPR 2014.014 calls for the determination of all-*trans* and *cis* isomers of lutein and β-carotene, as well as the separation of lutein from zeaxanthin. Selectivity was evaluated with visual inspection of chromatograms and by measuring the resolution of system suitability standard mixtures. Because apocarotenal is used as an internal standard, samples were prepared without internal standard to ensure there were no interfering peaks. To identify major *cis* isomers of α-carotene, β-carotene, and lutein, standard mixtures were isomerized by heating at 80°C for 2 h.

The separation of all-*trans*-lutein, *cis* isomers of lutein, zeaxanthin, and apocarotenal is shown in Figure **2016.13A**, whereas the separation of geometric isomers of α-carotene and β-carotene is shown in Figure **2016.13B**. Peak assignments were based on relative retention times from previous studies using C30 columns and methanol–MTBE as the mobile phase (11, 14–16). A chromatogram showing separation of lutein and β-carotene from lycopene is shown in Figure 1, and isomerized standard solutions showing major *cis* isomers of the carotenoids are shown in Figures 2 and 3. One of the minor *cis* isomers of β-carotene elutes before 15-*cis*-β-carotene, and this peak has a similar retention time to one

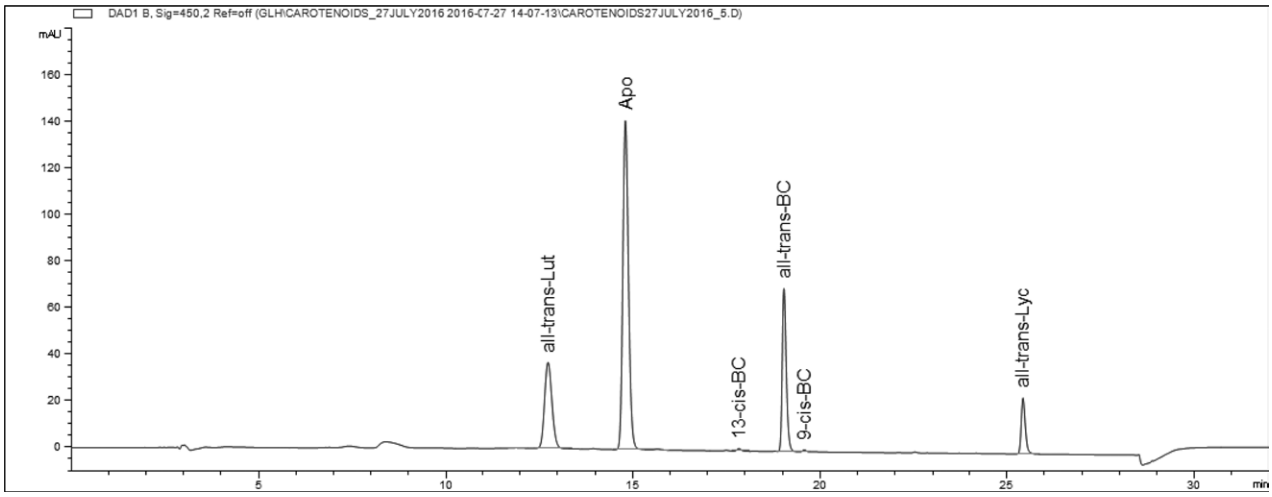

Figure 1. Chromatogram of calibration solution with addition of lycopene standard. Lut = lutein, Apo = apocarotenal, BC = β-carotene, and Lyc = lycopene.

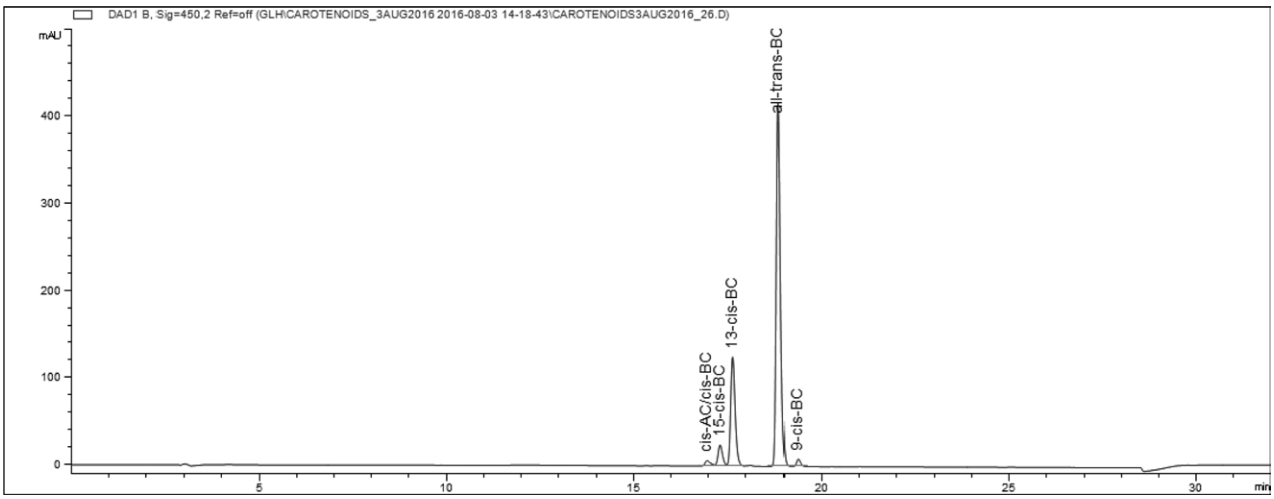

Figure 2. Chromatogram of heat-isomerized β-carotene solution. AC = α-carotene and BC = β-carotene.

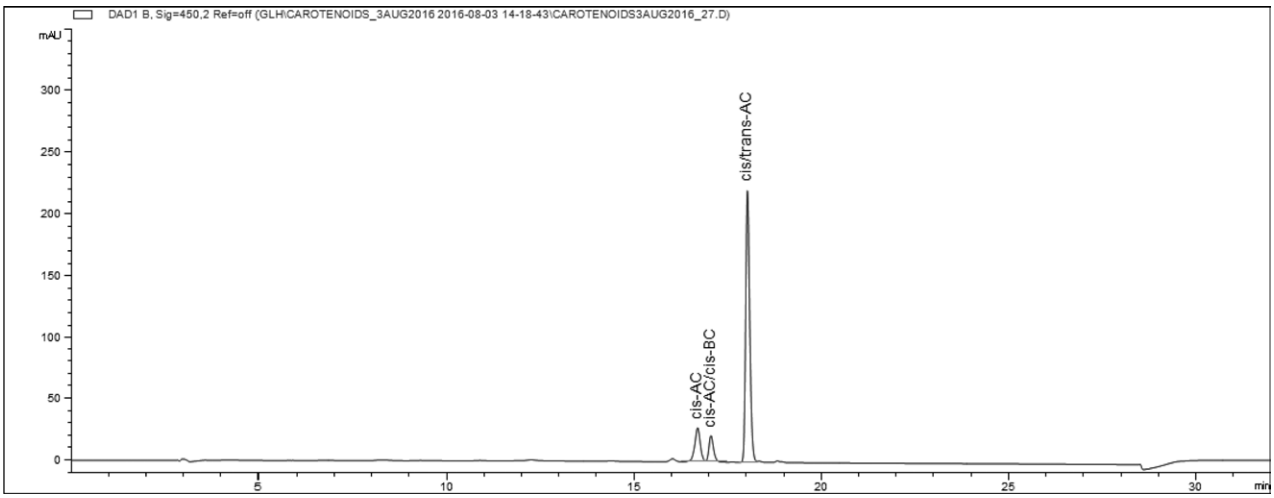

Figure 3. Chromatogram of heat-isomerized α-carotene solution. AC = α-carotene and BC = β-carotene.

of the major *cis* isomers of α-carotene. Although this could potentially cause error in the β-carotene calculation, even in a sample with high α-carotene (Figure **2016.13D**), the *cis*-α-carotene/*cis*-β-carotene peak accounted for only 5% of the total β-carotene peak area.

To test whether the all-*trans* isomers of lutein and β-carotene were isomerized during the sample preparation, chromatograms from spike and recovery experiments (n = 3) were used. Samples were spiked with all-*trans* carotenoid standards along with internal standard and carried through the preparation. No *cis* isomers of lutein were detected in the standard mixture, and none were detected in the spiked sample. In the β-carotene standard solution, *cis* isomers of β-carotene accounted for 3.4% of the total peak area in the standard mixture and 3.8% of the total β-carotene peak area in the spiked sample. This indicates that any isomerization of all-*trans*-lutein or β-carotene during the sample preparation is negligible.

Linearity

Linearity of the relative responses of analyte concentrations was measured using a five-point standard curve on 3 different days. Coefficients of determination, visual inspection, residuals, and relative errors of back-calculated concentrations were used for evaluation. Linearity of the internal standard was also tested. Regression lines for all-*trans*-lutein, all-*trans*-β-carotene, and apocarotenal are shown in Figure 4. Regression data for residuals and back-calculated concentrations are shown in Tables 1–3. The determination coefficients (R^2) for each curve were >0.999. The y-intercepts for all of the curves appeared insignificant; to test this assumption, sample calculations for all-*trans*-lutein and all-*trans*-β-carotene were performed by using both the y-intercept and forcing the y-intercept through zero. Two infant formulas were used: one with typical lutein and β-carotene concentrations and one with concentrations near the LOQ. The results (Tables 4 and 5) indicate that even for very low concentrations (3–4 µg/100 g) the difference between the two calculations was not more than 3%. Only when concentrations were near 1 µg/100 g did the calculations differ by as much as 9%. Based on these data, the y-intercept was forced through zero to simplify the calculations.

In accordance with SMPR 2014.014, all data for infant formula and adult nutritionals are presented on a reconstituted basis (as is for RTF liquids, 25 g powder/225 g reconstituted weight for powder samples, or 1:1 by weight for liquid concentrates). The ranges for lutein and β-carotene (4–240 µg/100 mL) correspond to approximately 0.8–45 µg/100 g for samples prepared for the lowest sample concentrations. With dilutions specified in the method, the range can be extended to approximately 2250 µg/100 g. This range extends beyond that of 1–1300 µg/100 g specified in the SMPR.

LOD/LOQ

The LOD and LOQ were extrapolated from the S/N calculated in ChemStation software (Agilent Technologies, Santa Clara, CA) when measuring analyte concentrations of 1.4–1.7 µg/100 g in spiked NIST SRM 1849a. The LOD was calculated as (3 × measured concentration)/(S/N). The LOQ was calculated as (10 × measured concentration)/(S/N). Results from three different spiked samples were averaged. The

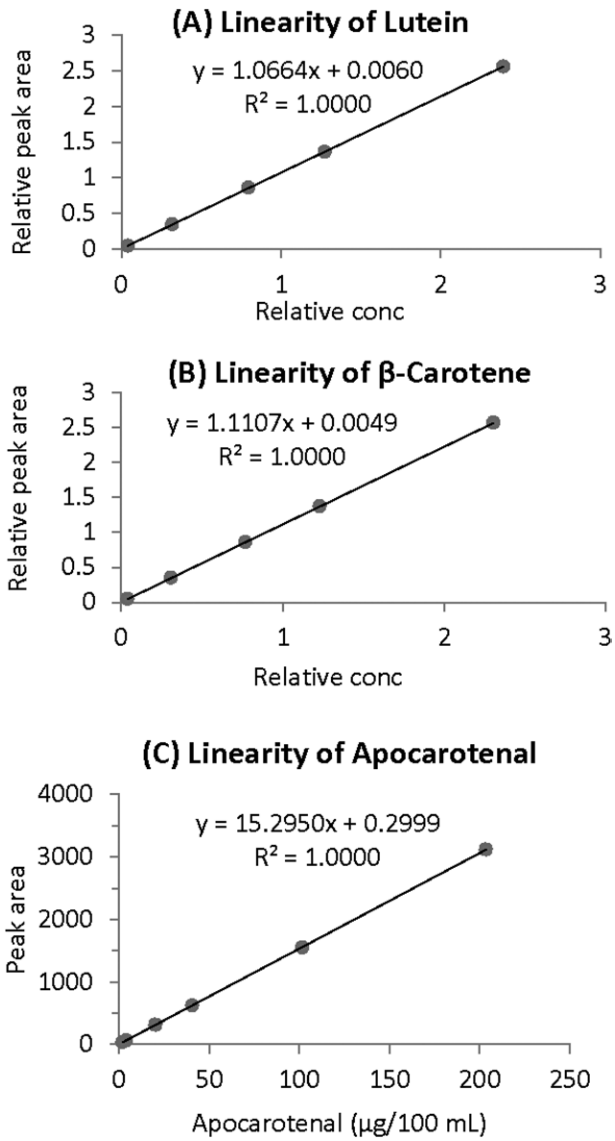

Figure 4. Linearity plot for (A) lutein, (B) β-carotene, and (C) apocarotenal.

determined LOQ for both lutein and β-carotene (Tables 6 and 7) meet the LOQ requirement of ≤1 µg/100 g in SMPR 2014.014.

Precision

Precision experiments were performed using the full SPIFAN sample kit, designed to represent current infant formulas and adult nutritional drinks on the market, in addition

Table 1. Residuals for the internal standard

Apocarotenal concn, µg/100 mL	Residual
203.5	4.119148067
101.8	–7.399430255
40.7	–3.177165248
20.4	1.435593088
4.07	2.222225757
2.04	2.79962859

Table 2. Residuals and error of back-calculated concentrations for all-*trans*-lutein standard curves

All-*trans*-lutein concn, µg/100 mL	Experiment 1		Experiment 2		Experiment 3	
	Residual	Error, %	Residual	Error, %	Residual	Error, %
235.9	0.000368916	0.01	–0.000497233	–0.02	–6.16301E-05	0.00
125.8	–0.003210486	0.24	–0.00122698	0.09	0.00221285	–0.16
78.6	0.00379152	–0.44	0.003308385	–0.38	–0.004609309	0.54
31.5	0.000817163	–0.24	0.000644834	–0.18	0.003230669	–0.93
3.93	–0.001767113	3.78	–0.002229005	4.65	–0.00077258	1.65

Table 3. Residuals and error of back-calculated concentrations for all-*trans*-β-carotene standard curves

All-*trans*-β-carotene concn, µg/100 mL	Experiment 1		Experiment 2		Experiment 3	
	Residual	Error, %	Residual	Error, %	Residual	Error, %
227.4	0.00125077	0.05	0.002290766	0.09	–0.001591824	–0.06
121.3	–0.002520458	–0.18	–0.003680882	–0.27	0.00318571	0.23
75.8	–0.000366823	–0.04	–0.00193471	–0.23	–0.000338007	–0.04
30.3	0.001615483	0.47	0.002244522	0.65	0.000226108	0.06
3.79	2.10278E-05	0.04	0.001080305	2.33	–0.001481986	–2.97

Table 4. Calculated concentrations (µg/100 g) for all-*trans*-lutein and all-*trans*-β-carotene in the control formula (milk-based infant formula) using the original standard curve and using zero as the *y*-intercept

Analyte	Experiment 1			Experiment 2			Experiment 3		
	Zero-intercept	Orig. curve[a]	Diff ., %[b]	Zero-intercept	Orig. curve	Diff., %	Zero-intercept	Orig. curve	Diff., %
Lutein	4.5	4.4	–1.9	4.4	4.3	–2.9	4.4	4.4	–1.4
β-Carotene	13.1	13.0	–0.9	13.0	13.0	–0.3	13.0	12.9	–0.5

[a] Orig. = Original.

[b] Diff. = Difference.

Table 5. Calculated concentrations (µg/100 g) for all-*trans*-lutein and all-*trans*-β-carotene in a FOS/GOS-fortified infant formula using the original standard curve and using zero as the *y*-intercept[a]

Analyte	Experiment 1			Experiment 2			Experiment 3		
	Zero-intercept	Orig. curve[b]	Diff., %[c]	Zero-intercept	Orig. curve	Diff., %	Zero-intercept	Orig. curve	Diff., %
Lutein	0.86	0.83	–3.5	0.86	0.78	–8.8	0.95	0.87	–8.3
β-Carotene	3.3	3.3	–1.6	3.3	3.3	–2.3	3.6	3.5	–2.6

[a] FOS/GOS = Fructooligosaccharide/galactooligosaccharide.

[b] Orig. = Original.

[c] Diff. = Difference.

Table 6. LOD and LOQ for lutein in spiked SRM 1849a

Sample	Measured concn, µg/100 g	S/N	Extrapolated LOD, µg/100 g	Extrapolated LOQ, µg/100 g
1	1.40	68.6	0.06	0.20
2	1.46	70.4	0.06	0.21
3	1.43	37.0	0.12	0.39
Average			0.08	0.27

Table 7. LOD and LOQ for β-carotene in spiked SRM 1849a

Sample	Measured concn, µg/100 g	S/N	Extrapolated LOD, µg/100 g	Extrapolated LOQ, µg/100 g
1	1.62	61.6	0.08	0.26
2	1.70	61.2	0.08	0.28
3	1.66	61.0	0.08	0.27
Average			0.08	0.27

to locally sourced samples that were fortified with lutein or β-carotene. All samples were prepared in triplicate, and the SPIFAN samples with substantial carotenoid concentrations were prepared at least 12 times (three replicates × 4 days). Intermediate precision (RSD_{iR}) was calculated as the RSD of all replicates. Repeatability (RSD_r) for triplicate samples was calculated as the RSD, and for larger samples sizes, it was estimated by performing a one-way analysis of variance on the intermediate precision data and calculating the SD from the within-day variance. Although SMPR 2014.014 does not specify criteria for intermediate precision, the requirements for repeatability and reproducibility are outlined in Table 8. Precision data are summarized in Tables 9 and 10. For all measurements in the range of 1–100 µg/100 g, RSD_r was ≤5.8% for lutein and ≤5.1 for β-carotene. For measurements >100 µg/100 g, RSD_r was ≤1.1% for lutein and ≤1.7 for β-carotene. RSD_{iR} ranged from 1.6 to 5.9% for lutein and from 0.7 to 6.3% for β-carotene for measurements >1 µg/100 g. For all measurements above 1 µg/100 g, the method met the precision requirements of the SMPR.

Table 8. Precision requirements from SMPR 2014.014

Sample concn, µg/100 g	Repeatability, %	Reproducibility, %
1–100	8	15
>100–1300	5	10

Accuracy

Because there is currently no Certified Reference Material for carotenoids in infant formula and adult nutritionals,

Table 9. Precision data for lutein in SPIFAN matrixes and select additional matrixes[a]

Matrix	SPIFAN code	*n*	*cis*-Lutein, µg/100 g	RSD_r, %	RSD_{iR}, %	*trans*-Lutein, µg/100 g	RSD_r, %	RSD_{iR}, %	Total lutein, µg/100 g	RSD_r, %	RSD_{iR}, %
Child Formula Powder, Placebo	00847RF00	3	<[b]	NA[c]	NA	<	NA	NA	<	NA	NA
Infant Elemental Powder, Placebo	00796RF	3	<	NA	NA	<	NA	NA	<	NA	NA
AN High-Protein RTF, Placebo[d]	00821RF00	3	<	NA	NA	3.3	3.8	NA	3.3	3.8	NA
AN High-Fat RTF, Placebo	00820RF00	3	2.0	3.8	NA	4.2	4.6	NA	6.2	4.1	NA
IF Milk-Based RTF, Placebo[e]	EV4H2Q	3	<	NA	NA	0.5	5.7	NA	0.6	4.8	NA
SRM 1849a Powder	CLC10-B	3	<	NA	NA	<	NA	NA	<	NA	NA
IF Milk-Based Partially Hydrolyzed Powder	410057652Z	3	<	NA	NA	<	NA	NA	<	NA	NA
IF Soy-Based Partially Hydrolyzed Powder	410057651Z	3	<	NA	NA	<	NA	NA	0.3	11.5	NA
Toddler Formula Milk-Based Powder	4052755861	12	3.7	1.2	2.2	21.9	3.6	3.8	25.5	3.1	3.4
IF Milk-Based Powder	4044755861	12	2.1	3.6	3.7	9.4	5.0	5.9	11.5	4.1	5.1
AN Powder	00859RF00	3	<	NA	NA	<	NA	NA	<	NA	NA
Child Formula Powder	00866RF00	3	<	NA	NA	<	NA	NA	<	NA	NA
IF Elemental Powder	00795RF	12	<	NA	NA	<	NA	NA	<	NA	NA
IF with FOS/GOS Powder[f]	50350017W1	12	0.3	12.3	14.3	0.9	2.9	5.2	1.2	3.4	4.2
IF Milk-Based Powder (control)	K16NTAV	39	1.6	3.3	5.5	4.5	1.2	1.6	6.1	1.6	2.3
IF Soy-Based Powder	E10NWZC	12	0.6	5.7	8.0	1.6	3.2	3.4	2.2	2.8	3.1
IF Milk-Based RTF	EV4H2R	3	<	NA	NA	0.5	6.2	NA	0.7	16.9	NA
AN High-Protein RTF	00730RF00	12	1.9	4.1	4.4	3.1	2.4	3.0	4.9	2.9	3.4
AN High-Fat RTF	00729RF00	3	1.7	5.8	NA	3.5	3.3	NA	5.2	3.2	NA
AN High-Lutein Powder	—[g]	3	21.4	0.3	NA	82.1	0.1	NA	104	0.2	NA
Chocolate Pediatric Nutritional RTF	—	3	13.0	0.4	NA	127	1.1	NA	140	1.0	NA
IF RTF	—	3	1.5	3.8	NA	3.9	1.4	NA	5.5	2.0	NA

[a] For powder samples, results were calculated using a factor of 25 g powder/225 g reconstituted weight.

[b] < = Below the LOQ.

[c] NA = Not applicable. RSDr not calculated for concentrations <LOQ; RSDiR not calculated if samples were all prepared on the same day.

[d] AN = Adult Nutritional.

[e] IF = Infant Formula.

[f] FOS/GOS = Fructooligosaccharide/galactooligosaccharide.

[g] — = Sample was sourced locally and was not part of the SPIFAN kit.

Table 10. Precision data for β-carotene in SPIFAN matrixes and select additional matrixes[a]

Formula	Code	*n*	*cis*-BC, µg/100 g[b]	RSD_r, %	RSD_{iR}, %	*trans*-BC, µg/100 g	RSD_r, %	RSD_{iR}, %	Total BC, µg/100 g	RSD_r, %	RSD_{iR}, %
Child Formula Powder, Placebo	00847RF00	3	0.3	0.7	NA[c]	0.6	2.6	NA	0.9	1.8	NA
Infant Elemental Powder, Placebo	00796RF	3	<[d]	NA	NA	<	NA	NA	<	NA	NA
AN High-Protein RTF, Placebo[e]	00821RF00	3	<	NA	NA	0.8	11.2	NA	0.8	11.2	NA
AN High-Fat RTF, Placebo	00820RF00	3	<	NA	NA	0.4	47.4	NA	0.6	48.5	NA
IF Milk-Based RTF, Placebo[f]	EV4H2Q	3	0.5	3.3	NA	0.4	2.2	NA	0.9	2.6	NA
SRM 1849a Powder	CLC10-B	3	<	NA	NA	<	NA	NA	<	NA	NA
IF Milk-Based Partially Hydrolyzed Powder	410057652Z	3	0.8	0.2	NA	1.0	2.0	NA	1.8	1.2	NA
IF Soy-Based Partially Hydrolyzed Powder	410057651Z	3	0.6	1.8	NA	0.6	6.7	NA	1.3	3.9	NA
Toddler Formula Milk-Based Powder	4052755861	12	6.5	1.0	2.4	7.4	0.7	2.9	13.9	0.8	2.2
IF Milk-Based Powder	4044755861	12	4.4	2.1	2.7	10.1	1.3	2.7	14.4	1.5	2.4
AN Powder	00859RF00	3	<	NA	NA	0.3	5.9	NA	0.3	5.9	NA
Child Formula Powder	00866RF00	3	0.3	15.3	NA	0.4	2.5	NA	0.7	5.4	NA
IF Elemental Powder	00795RF	12	7.3	0.3	0.7	15.3	0.7	0.7	22.7	0.5	0.6
IF with FOS/GOS Powder[g]	50350017W1	12	1.3	5.1	6.3	3.4	3.0	4.6	4.8	2.3	3.8
IF Milk-Based Powder (control)	K16NTAV	39	6.0	1.1	2.7	13.2	0.5	2.2	19.2	0.6	1.7
IF Soy-Based Powder	E10NWZC	12	6.5	2.6	2.3	11.0	2.0	1.8	17.5	2.2	1.9
IF Milk-Based RTF	EV4H2R	3	0.6	6.9	NA	0.4	4.8	NA	1.0	4.6	NA
AN High-Protein RTF	00730RF00	12	265	1.6	2.7	534	1.7	2.3	799	1.6	2.4
AN High-Fat RTF	00729RF00	3	<	NA	NA	0.3	7.7	NA	0.4	5.9	NA
AN High-Lutein Powder	—[h]	3	<	NA	NA	0.5	18.3	NA	0.5	18.3	NA
Chocolate Pediatric Nutritional RTF	—	3	<	NA	NA	<	NA	NA	<	NA	NA
IF RTF	—	3	9.1	0.3	NA	16.4	0.4	NA	25.5	0.4	NA

[a] For powder samples, results were calculated using a factor of 25 g powder/225 g reconstituted weight.

[b] BC = β-Carotene.

[c] NA = Not applicable. RSDr not calculated for concentrations <LOQ; RSDiR not calculated if samples were all prepared on the same day.

[d] < = Below the LOQ.

[e] AN = Adult Nutritional.

[f] IF = Infant Formula.

[g] FOS/GOS = Fructooligosaccharide/galactooligosaccharide.

[h] — = Sample was sourced locally and was not part of the SPIFAN kit.

accuracy was assessed by spike and recovery of all-*trans*-lutein and β-carotene in various matrixes. Spiking was done with either FloraGlo 5% lutein (DSM Nutritional Products, Basel, Switzerland), which was first dispersed in water and added to the sample in place of water, or with diluted standards added to the ISTD. Spiked and unspiked samples were measured in triplicate. Tables 11–17 show that recovery from spiked samples ranged from 92.3 to 105.5% for lutein and from 100.1 to 107.5% for β-carotene. The SMPR calls for 90–110% recovery from spiked samples, and all data met this requirement.

Conclusions

The data presented here were reviewed by the AOAC Expert Review Panel on SPIFAN Nutrient Methods at the AOAC INTERNATIONAL Annual Meeting in Dallas, TX, held in September 2016. The panel found that the data met the criteria

Table 11. Spike and recovery data for NIST SRM 1849a[a]

Analyte	Native level, µg/100 g	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %
Lutein	ND[b]	1.4	105.5	0.8	13.8	99.6	0.2
β-Carotene	0.2	1.3	107.5	0.9	13.6	103.2	0.3

[a] Samples were spiked with standards at the internal standard step of sample preparation.

[b] ND = Not detected.

Table 12. Spike and recovery data for NIST SRM 1849a[a]

Analyte	Native level, µg/100 g	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %
Lutein	ND[b]	34.8	92.3	0.8	68.3	94.0	1.9	135.3	97.6	1.6

[a] Samples were spiked with FloraGlo 5% lutein at the powder reconstitution step of sample preparation.

[b] ND = Not detected.

Table 13. Spike and recovery data for AN High-Protein RTF, Placebo[a]

Analyte	Native level, µg/100 g	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %
Lutein	3.3	62.7	96.7	0.8	148.8	100.1	0.3	297.6	101.0	0.3

[a] Samples were spiked with FloraGlo 5% lutein at the initial sample dilution step.

Table 14. Spike and recovery data for AN High-Protein RTF, Placebo[a]

Analyte	Native level, µg/100 g	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %
Lutein	3.3	486.4	101.1	0.5	708.8	101.2	0.5	1425.3	100.7	0.2

[a] Samples were spiked with FloraGlo 5% lutein at the initial sample dilution step.

Table 15. Spike and recovery data for Child Formula Powder, Placebo[a]

Analyte	Native level, µg/100 g	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %
Lutein	0.1	5.5	101.8	0.2	16.8	100.8	0.3
β-Carotene	0.6	5.3	107.2	0.2	16.2	104.2	0.3

[a] Samples were spiked with standards at the internal standard step of sample preparation.

Table 16. Spike and recovery data for Infant Elemental Powder, Placebo[a]

Analyte	Native level, µg/100 g	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %
Lutein	ND[b]	5.5	103.7	0.2	16.8	101.9	0.6
β-Carotene	0.1	5.3	104.1	0.2	16.2	103.5	0.6

[a] Samples were spiked with standards at the internal standard step of sample preparation.

[b] ND = Not detected.

Table 17. Spike and recovery data for Infant Formula RTF[a]

Analyte	Native level, µg/100 g	Spike, µg/100 g	Recovery, %	RSD, %	Spike, µg/100 g	Recovery, %	RSD, %
Lutein	3.9	6.2	98.7	0.4	18.4	98.6	0.1
β-Carotene	16.4	5.9	100.6	0.9	17.7	100.1	0.6

[a] Samples were spiked with standards at the internal standard step of sample preparation.

for specific carotenoids, (lutein and β-carotene) as stated in SMPR 2014.014, and recommended the method's approval as an AOAC First Action method. It is codified as *Official Method* **2016.13**.

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INFANT FORMULA AND ADULT NUTRITIONALS

Dual-Laboratory Validation of a Method for the Determination of Fructans in Infant Formula and Adult Nutritionals: First Action 2016.14

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Until recently, only two AOAC *Official Methods*[SM] have been available for the analysis of fructans: Method 997.08 and Method 999.03. Both are based on the analysis of the fructan component monosaccharides (glucose and fructose) after hydrolysis. The two methods have some limitations due to the strategies used for removing background interferences (such as from sucrose, α-glucooligosaccharides, and free sugars). The method described in this paper has been developed to overcome those limitations. The method is largely based on Method 999.03 and uses combined enzymatic and SPE steps to remove the interfering components without impacting the final analytical result. The method has been validated in two laboratories on infant formula and adult nutritionals. Recoveries were in the range of 86–119%, with most being in the range of 91–104%. RSD_r values were in the range of 0.7–2.6%, with one exception when the fructan concentration was close to the LOQ, resulting in an RSD_r of 8.9%. The performance is generally within the requirements outlined in the AOAC *Standard Method Performance Requirements* (SMPR® 2014.002), which specifies recoveries in the range of 90–110% and RSD_r values below 6%.

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The Expert Review Panel for SPIFAN Nutrient Methods 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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Inulin and fructooligosaccharides (FOS) are increasingly being used as health-enhancing ingredients in a diverse range of foods, feed, and pet food products. Both inulin and FOS pass the stomach and small intestine unchanged and are fermented in the large intestine where they stimulate the growth and/or activity of bacteria like lactobacilli and bifidobacteria, which may be beneficial to health (1, 2).

Historically, two different official AOAC Methods have been available for the determination of the total fructan content in food products: *Official Method*[SM] **997.08** (3) and *Official Method* **999.03** (4). The different underlying principles of the two methods result in each method having different advantages and disadvantages. Prior knowledge of the sample composition (content of sugars, maltodextrins, and starch) and of the fructan composition (presence of short-chain oligofructose) is necessary to be able to select the best approach.

In Method **997.08** (3), the free fructose and sucrose content must first be quantified chromatographically. In the next step, after enzymatic conversion of starch and maltodextrins, glucose is again measured chromatographically. In the third step, inulin/FOS and sucrose are completely converted into glucose and fructose, and then the released monosaccharides are determined chromatographically. The fructan content is calculated by subtracting the glucose, sucrose, and fructose content measured in steps 1 and 2 from the total fructose and glucose content measured in step 3. This implies that large corrections have to be made for samples containing large quantities of fructose,

glucose, sucrose, maltodextrin, and/or starch. The subtraction of two large values in order to calculate much lower inulin/FOS values generally results in less precise data with large SDs. Nevertheless, the method performs well when applied to products containing relatively low levels of interfering components.

The principle of Method **999.03** (4) differs from Method **997.08** (3), in that all monosaccharides present after combined α-glucanases and sucrase treatment are removed by converting them into alditols (via borohydride reduction). After enzymatic hydrolysis of the fructans, a colorimetric reducing sugar assay is then used to make the quantitative analysis. Fructans are thus accurately determined even in samples with high contents of monosaccharides, sucrose, maltodextrin, and/or starch, using relatively simple and inexpensive equipment. However, there is a drawback to this method: The reducing end groups of the fructan chains that do not terminate with a glucose (often referred to as Fm-type chains) are reduced into alditol end groups, which escape the analysis, resulting in low recoveries (e.g., the theoretical recoveries of fructobiose (F2) is only 50%, for F3, it is 67%; for F4, it is 75%, etc.). Fructan chains containing a terminal glucose (GFn-type chains) do not have a reducing end group, so these are recovered completely. FOS material prepared by depolymerization of inulin generally contains high amounts of F3 and F4, so total recovery can be below 80%. Although the method is not well suited to the analysis of samples containing FOS generated by partial hydrolysis of inulin, it is well suited to the analysis of long-chain fructans in a wide range of products and is quick and simple to apply.

Neither of the methods is optimal for the determination of fructans in infant formula or adult nutritionals. Therefore, the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) issued a call for new methods to meet the AOAC *Standard Method Performance Requirements* (SMPRs®) defined in SMPR 2014.002 (5). After consideration by the Expert Review Panel, two methods, including the one described here and Method **2016.06**, were considered to have acceptable performance.

Method **2016.06** (6, 7) is based on Method **999.03** (4) and on a method published by Cuany et al. (8), with a number of improvements. In Method **2016.06** (6, 7), a simplified sample preparation was introduced that reduces method turnaround time and, concomitantly, improves performance. The Cuany et al. method (8) required knowledge of the fructan type in the product to select appropriate correction factors (to correct for the "loss" of the terminal monomer of the fructan chains). Those correction factors are still required in Method **2016.06** (6, 7); however, a preanalysis step has been introduced to identify the fructan type and, thus, the appropriate correction factor, without the analyst needing (potentially confidential) recipe information.

The method described here has been designed to determine the fructan content without the need for ingredient-specific correction factors, thus avoiding the need for preanalysis (or knowledge of the fructan type in the product), and without interference from other components such as sucrose and free sugars. As in Method **999.03** (4), an enzyme mixture is used to hydrolyze sucrose and α-glucans to their constituent monosaccharides. Next, following the strategy of Cuany et al. (8), a graphitized carbon SPE column is used to eliminate the released glucose and fructose before the enzymatic hydrolysis of the fructans. The key difference is to avoid any borohydride treatment, thus avoiding the significant underestimation of Fm-type fructans. The method was codeveloped in two laboratories: Nestlé Research Centre (NRC) in Lausanne, Switzerland, and Carbohydrate Competence Centre of Eurofins (CCC) in Heerenveen, The Netherlands. Single-laboratory validations (SLVs) were performed independently in both centers.

AOAC Official Method 2016.14
Fructans in Infant Formula and Adult Nutritionals
HPAEC-PAD
First Action 2016

A. Principle

Samples are reconstituted in water (if required) and further diluted until the concentration of fructan in solution is such that after hydrolysis, the fructose and glucose concentration is within the range covered by the standard curve. The diluted sample is treated with a mixture of sucrase and α-glucanases to hydrolyze sucrose and α-glucans, respectively, releasing their constituent monosaccharides. The sample is passed through an SPE cartridge packed with graphitized carbon. Salts and monosaccharides pass through and are washed away, while the fructans are retained. Fructans are released from the column using an acetonitrile solution. The released fructans are hydrolyzed with an inulinase mixture, and the released glucose and fructose are analyzed by high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection (PAD). The fructan content is calculated by summing the glucose and 0.9× the fructose content measured. In some matrixes containing low amounts of fructans, a blank correction may be necessary and can be applied. In this study, blank corrections were required only at the lowest fructan concentration (0.03 g/100 g) and only for a few products. For unknown matrixes containing fructan concentrations below 0.1 g/100 g, it is advisable to check whether the blank correction is required.

B. Materials

Samples from the SPIFAN II SLV Kit were provided by Covance (Madison, WI) and are listed in Table **2016.14A**. In addition, two infant formulas from Nestlé (Vevey, Switzerland) were included in the SLV performed at NRC. Fructan ingredients used for spiking experiments were Orafti® P95 and Orafti HP (both from Beneo, Tienen, Belgium) and NutraFlora® P-95 (Ingredion, Inc., Westchester, IL).

The samples were stored in the original package in a dry place and protected from light until the moment of use. According to the instructions for this SLV Kit and the AOAC *Standard Method Performance Requirements* (SMPRs®) defined in SMPR 2014.002 (5), all powder products except SRM 1849a were reconstituted by dissolving 25 g powder in 200 g water. The SRM 1849a was weighed directly or reconstituted by dissolving 10 g in 90 g water.

In this validation study, three different standards of pure fructan ingredients were used:

(1) Orafti HP, a long-chain inulin ingredient.

(2) Orafti P95, a hydrolyzed inulin ingredient consisting of both GFn and Fm constituents.

Table 2016.14A. Contents of the SPIFAN SLV sample kit

Sample No.	Product description	Lot No.	Matrix type	Country of origin
	Placebo products			
1	Child Formula Powder	00847RF00	Powder	United States
2	Infant Elemental Powder	00796RF	Powder	United States
3	Adult Nutritional RTF, High-Protein	00821RF00	Liquid	United States
4	Adult Nutritional RTF, High-Fat	00820RF00	Liquid	United States
5	Infant Formula RTF, Milk-Based	EV4H2Q	Liquid	United States
	Fortified products			
6	SRM 1849a	CLC10-b	Powder	United States
7	Infant Formula Powder, Partially Hydrolyzed Milk-Based	410057652Z	Powder	United States
8	Infant Formula Powder, Partially Hydrolyzed Soy-Based	410457651Z	Powder	United States
9	Toddler Formula Powder, Milk-Based	4052755861	Powder	Ireland
10	Infant Formula Powder, Milk-Based	4044755861	Powder	Ireland
11	Adult Nutritional Powder, Low-Fat	0859RF00	Powder	United States
12	Child Formula Powder	00866RF00	Powder	United States
13	Infant Elemental Powder	00795RF	Powder	United States
14	Infant Formula Powder, FOS/GOS-Based	50350017W1	Powder	Switzerland
15	Infant Formula Powder, Milk-Based	K16NTAV	Powder	United States
16	Infant Formula Powder, Soy-Based	E10NWZC	Powder	United States
17	Infant Formula Powder RTF, Milk-Based	EV4H2R	Liquid	United States
18	Adult Nutritional RTF, High-Protein	00730RF00	Liquid	United States
19	Adult Nutritional RTF, High-Fat	0729RF00	Liquid	United States

(3) NutraFlora P-95, a short-chain FOS based on enzymatic sucrose elongation, mainly consisting of GF2, GF3, and GF4 constituents.

The purity of these standards was established by analysis according to Method **997.08** (3).

C. Apparatus

(a) *Analytical balance*.—Weighing to ±0.1 mg (Mettler-Toledo, Greifensee, Switzerland).

(b) *pH Meter*.—Reading 0.1 pH (Metrohm, Herisau, Switzerland).

(c) *Microtubes*.—2 mL (Eppendrof, Hamburg, Germany).

(d) *Water bath*.—80 ± 1°C (Thermo Fisher Scientific, Waltham, MA).

(e) *Water bath*.—40 ± 1°C (Thermo Fisher Scientific).

(f) *Centrifuge*.—For 2 mL microtubes able to operate at 10 000 × *g* (Eppendorf).

(g) *Micropipets with tips (0.1–1 mL)*.—Socorex IsbA S.A. (Ecublens, Switzerland).

(h) *Vortex mixer*.—Scientific Industries (Bohemia, NY).

(i) *Single-use plastic pipets, 5 and 10 mL*.—Becton, Dickinson & Co. (Franklin Lakes, NJ).

(j) *Single-use syringes, 2 mL*.—Becton, Dickinson & Co.

(k) *Syringe membrane filters, nylon, 0.2 µm, Ø 13 mm*.—Teknokrama (Barcelona, Spain).

(l) *Graphitized carbon (100 mg) SPE columns*.—Sigma-Aldrich (St. Louis, MO).

(m) *Membrane filter nylon, 0.2 µm, Ø 4.7 cm*.—Merck-Millipore (Merck KGaA, Darmstadt, Germany).

(n) *Analytical column*.—CarboPac PA20 (150 × 3 mm, 6.5 µm) or CarboPac PA1 (250 × 2 mm, 10 µm) with guard (50 × 2 mm, 10 µm; Thermo Fisher Scientific).

(o) *HPAEC–PAD system*.—Including an eluent sparging system; gradient pump; autosampler; column compartment able to maintain a temperature of 20 ± 0.5°C (for the PA1 column) or 30 ± 0.5°C (for the PA20 column); electrochemical detector working in PAD mode; and isocratic pump for postcolumn delivery.- all Thermo Fisher Scientific.

D. Chemicals and Reagents

(a) *Deionized water*.—18 megaohm-cm Milli-Q purified, or equivalent.

(b) *Maleic acid, puriss., >99%*.—Sigma-Aldrich.

(c) *Acetonitrile gradient grade for LC*.—Merck-Millipore.

(d) *Acetic acid glacial 100% anhydrous, guaranteed reagent for analysis*.—Merck-Millipore.

(e) *Potassium cyanohexaferrate(II) trihydrate*.—Optional (Merck-Millipore).

(f) *Zinc acetate*.—Optional (Merck-Millipore).

(g) *Trifluoroacetic acid (TFA)*.—Sigma-Aldrich.

(h) *Hydrochloric acid, 1 M*.—Merck-Millipore.

(i) *Sodium acetate, anhydrous*.—Sigma-Aldrich.

(j) *Sodium hydroxide 50% (w/w)*.—(J.T. Baker, Deventer, The Netherlands). Used for the preparation of LC eluents. To avoid production of carbonate, the bottle should be opened for the minimum time necessary to avoid exposure to air. The solution should no longer be used for eluent preparation after about twothirds of the bottle is empty (because the remaining solution will contain too much carbonate). The remaining solution can be used for other applications in the laboratory (including preparation of the solution for postcolumn addition).

(k) *Sodium hydroxide pellets*.—Merck-Millipore.

(l) *Helium for eluent sparging*.

(m) *Sodium chloride*.—Merck-Millipore.

(n) *Sodium azide*.—For use only with the PA1 column for HPAEC–PAD (Sigma-Aldrich).

(o) *D-(−)-fructose, >99%*.—Sigma-Aldrich.

(p) *D-(+)-glucose, ≥99.5%*.—Sigma-Aldrich.

(q) *Chitobiose*.—Elicityl S.A. (Crolles, France).

(r) *Mixture of highly purified sucrase, β-amylase, pullulanase, and maltase (from Fructan Assay Kit; K-FRUC)*.—Megazyme (Bray, Ireland).

(s) *Mixture of highly purified exo-inulinase and endo-inulinase (from K-FRUC)*.—Megazyme.

E. Preparation of Reagents

(a) *Sodium maleate buffer (100 mM, pH 6.5)*.—Into a large beaker (>1000 mL), weigh 11.6 g maleic acid and dissolve with 900 mL water (using a magnetic stirrer). Adjust the pH to 6.5 with 1 M sodium hydroxide solution. Transfer the solution to a 1000 mL volumetric flask and dilute to the mark with water. (Stored at 6 ± 2°C, this solution is stable for 6 months.)

(b) *Sodium acetate buffer (100 mM, pH 4.5)*.—Into a large beaker (>1000 mL) containing 900 mL demineralized water, pipet 5.8 mL glacial acetic acid. Adjust to pH 4.5 with 1 M sodium hydroxide solution. Transfer the solution to a 1000 mL volumetric flask and dilute to the mark with water. (Stored at 6 ± 2°C, this solution is stable for 6 months.)

(c) *Chitobiose solution (600 µg/mL)*.—Into a 25 mL volumetric flask, weigh 15 mg chitobiose and dilute to the mark with water. (Stored at 6 ± 2°C, this solution is stable for 1 week, or aliquot and store at −20 ± 2°C for up to 12 months.)

(d) *Glucose stock solution (5 mg/mL)*.—Into a 25 mL volumetric flask, weigh 125 mg glucose and dilute to the mark with water. (Stored at 6 ± 2°C, this solution is stable for 1 week, or aliquot and store at −20 ± 2°C for up to 12 months.)

(e) *Fructose stock solution (10 mg/mL)*.—Into a 25 mL volumetric flask, weigh 250 mg fructose and dilute to the mark with water. (Stored at 6 ± 2°C, this solution is stable for 1 week, or aliquot and store at –20 ± 2°C for up to 12 months.)

(f) *Sodium hydroxide (1 M)*.—Dissolve 40 ± 1 g sodium hydroxide pellets in 500 mL water in a 1000 mL volumetric flask. After cooling down to room temperature, dilute to the mark with demineralized water and mix well. (Stored at 22 ± 5°C, this solution is stable for 6 months).

(g) *Carrez I solution*.—Dissolve 106 g potassium hexacyanoferrate(II) trihydrate in 1000 mL demineralized water and store in a brown bottle (optional reagent; stored at 22 ± 5°C, this solution is stable for 1 month).

(h) *Carrez II solution*.—Dissolve 220 g zinc acetate in 900 mL demineralized water in a 1000 mL volumetric flask and then add 29 mL glacial acetic acid. Dilute to the mark with demineralized water and homogenize (optional reagent; stored at 22 ± 5°C, this solution is stable for 1 month).

(i) *Sodium azide solution (0.5%)*.—Dissolve 1 g sodium azide in 200 mL demineralized water (optional reagent needed only for the LC method on the PA1 column; stored at 22 ± 5°C, this solution is stable for 12 months).

(j) *Sucrase/β-amylase/pullulanase/maltase*.—Dissolve the contents of the vial containing powdered sucrase, β-amylase, pullulanase, and maltase in 22.0 mL sodium maleate buffer (100 mM, pH 6.5). Mix well and divide into aliquots of 2.0 mL each and store frozen at –20°C in polypropylene tubes until use. (Stored at –20 ± 2°C, this solution is stable for 12 months.) *Note*: For the development and validation of this method, the preprepared enzyme mixture available in the Megazyme kit, K-FRUC, was used. When enzymes from another source are used, it is imperative to ensure that the enzyme mixture will completely hydrolyze any sucrose in the product without hydrolyzing the fructan. This can be checked by performing an analysis with sucrose as an analyte and with a pure fructan as an analyte. No fructan should be measured when sucrose is analyzed, and >90% recovery should be achieved when a pure fructan is analyzed.

(k) *Fructanase (exo-inulinase/endo-inulinase)*.—Dissolve the contents of the vial containing powdered exo-inulinase and endo-inulinase in 22.0 mL sodium acetate buffer (100 mM, pH 4.5). Mix well and divide into aliquots of 2.0 mL each and store frozen at –20°C in polypropylene tubes until use. (Stored at –20 ± 2°C, this solution is stable for 12 months.) *Note*: For the development and validation of this method, the preprepared enzyme mixture available in the Megazyme kit, K-FRUC, was used. When enzymes from another source are used, it is imperative to ensure that the enzyme mixture used will completely hydrolyze the fructan without hydrolyzing any other glucose- or fructose-containing oligo- or polysaccharide that may be present after treatment with the sucrase mixture in **E(j)**.

(l) *Wash solution for graphitized carbon column, 0.1% TFA–80% acetonitrile (v/v)*.—Into a 100 mL volumetric flask, add 80 mL acetonitrile (HPLC grade) and 100 µL TFA. Dilute to the mark with water. (Stored at 22 ± 5°C, this solution is stable for 6 months.)

(m) *Sodium chloride solution (1 M) for graphitized carbon column*.—Into a 100 mL volumetric flask, weigh 5.8 g sodium chloride and dissolve with 90 mL demineralized water. Dilute to the mark with water. (Stored at 22 ± 5°C, this solution is stable for 6 months.)

(n) *Elute solution for graphitized carbon column, 0.05% TFA–25% acetonitrile (v/v)*.—Into a 100 mL volumetric flask, add 25 mL acetonitrile (HPLC grade) and 50 µL TFA. Dilute to the mark with water. (Stored at 22 ± 5°C, this solution is stable for 6 months.)

F. Mobile Phase Preparation (Using CarboPac PA 20) Performed at NRC

(a) *Eluent A for PA20 column: 300 mM sodium hydroxide solution*.—Into an HPLC bottle, introduce 985 mL deionized water, and degas with helium for 20 min. Add 15.6 mL sodium hydroxide solution (50%). Degas with helium for 20 min and keep under a blanket of helium until, and during, use. (Stored at 22 ± 5°C under a blanket of helium, this solution is stable for 1 week.)

(b) *Eluent B for PA20 column: Milli-Q water*.—Into an HPLC bottle, introduce 2000 mL water, and degas with helium for 20 min. Thereafter, keep under a blanket of helium until, and during, use. (Stored at 22 ± 5°C under a blanket of helium, this solution is stable for 2 days.)

(c) *Eluent C for PA20 column: 500 mM sodium acetate–150 mM sodium hydroxide solution*.—Into a 1000 mL volumetric flask, weigh 41.0 g anhydrous sodium acetate and dissolve with 800 mL water by mixing. Dilute to the mark with

water, and filter on a 0.20 µm nylon membrane filter into an HPLC bottle. Degas with helium for 20 min and then add (using a single-use plastic pipet) 7.8 mL 50% (w/w) NaOH solution. Swirl gently to mix, and sparge with helium for another 15 min. Thereafter, keep under a blanket of helium until, and during, use. (Stored at 22 ± 5°C under a blanket of helium, this solution is stable for 1 week.)

(d) *Postcolumn addition reagent: 300 mM sodium hydroxide*.—Into an HPLC bottle, introduce 985 mL water and add 15.6 mL NaOH 50% solution (using a single-use plastic pipet). Swirl the solution gently to mix. Degas with helium for 20 min and keep under a blanket of helium until, and during, use. (Stored at 22 ± 5°C, this solution is stable for 1 month.)

G. Mobile Phase Preparation (Using CarboPac PA 1) Performed at CCC

(a) *Eluent A for PA1 column: 200 mM sodium hydroxide solution*.—Weigh 3846 ± 5 g deionized water in the eluent bottle, and degas with helium for 20 min. Add 40 mL sodium hydroxide solution (50%). Degas with helium for 20 min and keep under a blanket of helium until, and during, use. (Stored at 22 ± 5°C under a blanket of helium, this solution is stable for 1 week.)

(b) *Eluent B for PA1 column*: *Milli-Q water with sodium azide.*—Fill a 4 L eluent bottle with 3900 mL carbonate-free Milli-Q water. Add 100 mL 0.5% sodium azide solution. Degas with helium for 20 min and keep under a blanket of helium until, and during, use. (Stored at 22 ± 5°C under a blanket of helium, this solution is stable for 1 week.)

(c) *LC eluent C for PA1 column: 1 M sodium acetate solution*.—Into a 1000 mL volumetric flask, weigh 82.0 g anhydrous sodium acetate and dissolve with 800 mL water by mixing. Dilute to the mark with deionized water and filter on a 0.20 µm nylon membrane filter into an eluent bottle. Degas with helium for 20 min and keep under a blanket of helium until, and during, use. (Stored at 22 ± 5°C under a blanket of helium, this solution is stable for 1 week.)

(d) *LC postcolumn addition reagent: 300 mM sodium hydroxide*.—Into an HPLC bottle, introduce 985 mL water and add 15.6 mL NaOH 50% solution (using a single-use plastic pipet). Swirl the solution gently to mix. Degas with helium for 20 min and keep under a blanket of helium until, and during, use. (Stored at 22 ± 5°C, this solution is stable for 1 month.)

H. Preparation of Standards

Using volumetric flasks, prepare a six-level standard curve by diluting the glucose stock solution (5 mg/mL) and the fructose stock solution (10 mg/mL) to the final volume with deionized water, as described in Table **2016.14B**.

Treat each of the six solutions of standards as follows: Into a microtube, transfer 200 µL standard solution and add 200 µL water and 100 µL chitobiose internal standard solution. Next, transfer a 400 µL aliquot of this solution to another microtube and add 1200 µL SPE elute solution. To a 700 µL aliquot of this mixture, add 300 µL sodium acetate buffer. Mix well and then centrifuge at 10 000 × *g*. Transfer a 900 µL portion of the supernatant into a vial suitable for the instrument autosampler.

Table 2016.14B. Dilution scheme for the preparation of the standard curve

Standard curve level	Fructose stock solution vol., µL	Glucose stock solution vol., µL	Final vol., mL	Fructose concn, µg/mL	Glucose concn, µg/mL
1	200	40	100	20	2
2	400	200	20	200	50
3	800	400	20	400	100
4	1200	600	20	600	150
5	1600	800	20	800	200
6	2000	1000	20	1000	250

I. Sample Preparation

(a) *For analysis of products on a ready-to-feed (RTF) basis*.—Reconstitute powder or liquid concentrates according to instructions. For example, weigh 25 g infant formula powder into a bottle and add water (200 g). Mix well at room temperature, and record the final weight.

(b) *For reconstituted products (as prepared above) or for products that are sold as RTF*.—Weigh 9 g into a 50 mL volumetric flask and add 30 mL water. Confirm that the pH is between 5 and 9 (adapt pH using 1 M hydrochloric acid or 1 M sodium hydroxide solution, if needed) and place in a water bath at 80°C with constant agitation for 20 min. After cooling, dilute to the mark with water (this is Solution A). Alternative dilutions schemes have also been applied (*see* Table **2016.14C**).

(c) *For analysis of powder products without prior reconstitution*.—Weigh 1 g powder into a 50 mL volumetric flask. Add 30 mL water and confirm that the pH is between 5 and 9 (adapt pH using 1 M hydrochloric acid or sodium 1 M hydroxide solution, if needed). Heat at 80°C with constant agitation for 20 min. Cool to room temperature and dilute to the mark with water (this is Solution A).

The solutions prepared above are further diluted, depending on the expected fructan content, following the guidelines in Table **2016.14C**, and the resulting solution is Solution B.

(d) *Hydrolysis of sucrose and α-glucans*.—Transfer 200 µL Solution B into a 1.5 mL microtube and add 100 µL chitobiose solution (600 µg/mL) and 200 µL sucrose/maltase/amylase/pullulanase enzyme mixture. Mix well and incubate at 40°C for 90 min.

(e) *Optional Carrez clarification*.—Performed at CCC but not at NRC. Add 10 µL Carrez I solution to the sample and mix well. Next, add 10 µL Carrez II solution and mix again. Centrifuge at 10 000 × *g* for 10 min, and use the supernatant for the next step.

(f) *Removal of monosaccharides (CCC procedure)*.—Prepare the graphitized carbon SPE column as follows:

(1) Flush with 3 × 400 µL wash solution.

(2) Flush with 3 × 400 µL water.

(3) Perform the following steps under gravity (i.e., without applying vacuum or positive pressure):

(a) Apply 400 µL enzyme-treated solution.

(b) Wash with 1 × 400 µL sodium chloride solution (1 M).

(c) Wash with 2 × 800 µL sodium chloride solution (1 M).

Table 2016.14C. Possible schemes for sample dilution depending on expected fructan content

Expected fructan content, g/100 g		Preparation of Solution A[a]			Dilution to Solution B		
Powder	RTF	Powder weight, g	RTF weight, g	Final vol., mL	Solution A vol., mL	Final vol., mL	Dilution factor
Used at NRC							
<4.5	<0.5	1	9	50	No dilution	No dilution	1
4.5–9	0.5–1.0	1	9	50	5	10	2
9–27	1.0–3.0	1	9	50	5	25	5
27–36	3.0–4.0	1	9	50	5	50	10
36–45	4.0–5.0	1	9	50	5	100	20
Used at CCC							
<1	0.03–5.0	4	4	100	No dilution	No dilution	1
1–5	NA[b]	1	NA	100	No dilution	No dilution	1
5–10	NA	1	NA	100	0.1	0.2	2
10–20	NA	1	NA	100	1	5	5
20–100	NA	1	NA	100	0.25	5	20

[a] Solution A is prepared by either diluting the indicated powder weight to the final volume or diluting the indicated weight of the RTF product to the final volume.

[b] NA = Not Applicable.

(d) Wash with 5 × 800 µL water.

(e) Elute the fructans using 5 × 400 µL elute solution.

(f) Mix eluates from the SPE cartridge well.

(g) *Removal of monosaccharides (NRC procedure).*—Prepare the graphitized carbon SPE column as follows:

(1) Flush with 3 × 400 µL wash solution.

(2) Flush with 3 × 400 µL water.

(3) Perform the following steps under gravity (i.e., without applying vacuum or positive pressure):

(a) Apply 400 µL enzyme-treated solution.

(b) Wash with 2 × 1000 µL sodium chloride solution (1 M).

(c) Wash with 4 × 1000 µL water.

(d) Elute the fructans into a 2 mL microtube using 3 × 400 µL elute solution.

(e) Apply a little positive pressure to eliminate all solution from the column.

(f) Mix eluates from the SPE cartridge well.

(h) *Hydrolysis of fructans (CCC procedure).*—Transfer a 1000 µL portion of the eluate from the SPE cartridge into a microtube and add 350 µL sodium acetate buffer (100 mM, pH 4.5) and 100 µL inulinase mixture. Mix well and incubate at 40°C for 40 min.

(i) *Hydrolysis of fructans (NRC procedure).*—To the eluate from the SPE cartridge, add 300 µL sodium acetate buffer (100 mM, pH 4.5). Transfer a 700 µL portion of this solution into a microtube (marked "sample") and add 100 µL inulinase mixture. Into a second microtube (marked "blank"), transfer a 700 µL portion of the eluate and add 100 µL sodium acetate buffer (100 mM, pH 4.5). (The blank is necessary only for some matrixes containing low amounts of fructans and may be skipped if it has already been established that it is not needed for a given matrix). For all tubes, mix well and incubate at 40°C for 40 min.

(j) After cooling, centrifuge at 10000 × *g* and then transfer a 700 µL portion of the supernatant into a vial suitable for the instrument autosampler, or pass the hydrolysate through a 0.2 µm syringe filter into the autosampler vial.

J. Chromatographic Conditions

(a) *Using PA1 (CCC Method).*—The HPAEC–PAD system is equipped with the CarboPac PA1 guard (2 × 50 mm, 10 µm) and analytical columns (2 × 250 mm, 10 µm), or equivalent, connected in series. The columns are held at 20°C, and the injection volume is 20 µL. Sodium hydroxide (300 mM) is added postcolumn (before PAD) at a flow rate of 0.13 mL/min. Fructose and glucose are separated using the gradient described in Table **2016.14D**. Carbohydrates are detected by pulsed amperometry using the quadruple waveform described in Table **2016.14E**.

(b) *Using PA20 (NRC Method).*—The HPAEC–PAD system is equipped with the CarboPac PA20 (3 × 150 mm, 6.5 µm) column, or equivalent. The column is held at 30°C, and the injection volume is 25 µL. Sodium hydroxide (300 mM) is added postcolumn (before PAD) at a flow rate of 0.2 mL/min. Fructose and glucose are separated using the gradient described in Table **2016.14F**. Carbohydrates are detected by pulsed amperometry using the quadruple waveform described in Table **2016.14E**.

K. Calibration and Calculations

Use bracketed calibration by injecting three standards followed by 10 samples, and repeating this process (e.g., inject standards at levels 1, 3, and 5 and then 10 samples; inject standards at levels 2, 4, and 6 and then 10 samples; inject standards 1, 3, 5, etc.). For each analyte (glucose and fructose), use the instrument software to plot a six-point standard curve of (instrument response for analyte)/(instrument response for internal standard) against the

Table 2016.14D. HPAEC–PAD gradient for PA1 column, or equivalent

Time, min	Flow, mL/min	A, %[a]	B, %[b]	C, %[c]
0.0	0.25	7.5	92.5	0.0
13.0	0.25	7.5	92.5	0.0
14.1	0.25	25.0	75.0	0.0
20.0	0.25	25.0	75.0	0.0
21.0	0.25	40.0	30.0	30.0
28.0	0.25	40.0	30.0	30.0
30.0	0.25	4.0	60.0	0.0
31.0	0.25	7.5	92.5	0.0
43.0	0.25	7.5	92.5	0.0

[a] A = 200 mM NaOH.

[b] B = Water.

[c] C = 1 M NaOAc.

Table 2016.14E. Quadruple waveform for carbohydrate detection

Time, s	Voltage, V	Gain region
0.00	+ 0.10	Off
0.20	+ 0.10	On
0.40	+ 0.10	Off
0.41	−2.00	Off
0.42	−2.00	Off
0.43	+ 0.60	Off
0.44	−0.10	Off
0.50	−0.10	Off

concentration of the analyte in the standard. Fit a quadratic curve to the data without forcing through zero. Use the calibration curve to calculate the glucose and fructose concentration in Solution B.

Calculate the fructan concentration in the sample as follows:

$$C_G = C_{GB} \times D \times (V/m) \times 0.0001$$

$$C_F = C_{FB} \times D \times (V/m) \times 0.0001$$

$$TF = (C_F \times 0.9) + C_G$$

where C_G = the concentration of glucose (g/100 g) released from fructan; C_{GB} = the concentration (µg/mL) of glucose in Solution B; D = the dilution factor between Solution A and Solution B (*see* Table **2016.14C**); V = the total volume (mL) of Solution A; m = the amount (g) of sample weighed to prepare Solution A; 0.0001 = the factor to convert analyte concentration in solution (µg/mL) to analyte concentration in sample (g/100 g); C_F = the concentration (g/100 g) of fructose released from fructan; C_{FB} = the concentration (µg/mL) of fructose in Solution B; 0.9 = the factor to correct for uptake of water during fructan hydrolysis; and TF = the total fructan concentration (g/100 g) in the sample.

Table 2016.14F. HPAEC–PAD gradient for PA20 column, or equivalent

Time, min	Flow, mL/min	A, %[a]	B, %[b]	C, %[c]
0.0	0.5	2	98	0
17.0	0.5	2	98	0
17.1	0.5	0	0	100
22.0	0.5	0	0	100
22.1	0.5	100	0	0
27.0	0.5	100	0	0
27.1	0.5	2	98	0
33.0	0.5	2	98	0

[a] A = 300 mM NaOH.

[b] B = Water.

[c] C = 500 mM NaOAc + 150 mM NaOH.

For samples with low fructan content requiring the blank correction, adapt the above equations as follows:

$$C_G = (C_{GB} - C_{G0}) \times D \times (V/m) \times 0.0001$$

$$C_F = (C_{FB} - C_{F0}) \times D \times (V/m) \times 0.0001$$

where C_{G0} = the concentration (µg/mL) of glucose in blank Solution B; and C_{F0} = the concentration (µg/mL) of fructose in blank Solution B.

L. Validation Design

Table **2016.14G** summarizes the main requirements described in SMPR 2014.002 (5) for the determination of fructans in infant formula and adult nutritionals. The SLVs were designed to test the method against those requirements. Reproducibility could not be assessed with only two laboratories; however, intermediate reproducibility was assessed and provided a guide as to whether the reproducibility targets might be achievable.

(a) *Calibration fit*.—The calibration fit was assessed at NRC by injecting calibration solutions at eight different concentrations (2–300 µg/mL for glucose and 20–1100 µg/mL for fructose), all containing the same amount of chitobiose internal standard. Each level was prepared in triplicate. The ratio of analyte-to-chitobiose peak areas was plotted against analyte concentration, and a quadratic model was used to fit the data. The relative residuals were calculated and plotted against analyte concentration. At CCC, the same approach was taken but using 12 different concentrations (0.051–21.78 µg/mL for glucose and 0.887–179 µg/mL for fructose).

(b) *LOD and LOQ*.—The LOD and LOQ were assessed in slightly different ways in the two laboratories. At CCC, an infant formula containing no fructans was spiked with a low level of fructan (just above the desired LOQ of 0.03 g/100 g) and analyzed 10 times (this was performed on 2 different days, with a 3-month interval in between). The SD of the results was multiplied by 3 to estimate the LOD and by 10 to estimate the LOQ. At NRC, a different infant formula was selected. It was also a blank formula, but when analyzed, minor signals at the retention times of glucose and fructose could be observed. Those signals were treated as if they actually originated from fructan, and the amount of fructan they represented was measured 14 times (7 days in duplicate). The LOD and LOQ were then calculated by taking the mean

Table 2016.14G. SMPRs for the determination of fructans in infant formula and adult nutritionals[a]

Parameter	Value
Analytical range, g/100 g[b]	0.03–5.0
LOQ, g/100 g[b]	≤0.03
RSD_r, %	≤6
RSD_R, %	≤12
Recovery, %	90–110

[a] SMPR 2014.002 (5).

[b] Concentrations apply to the product as consumed (i.e., reconstituted powders or concentrates, or as is for RTF products).

fructan content and adding 3 times the SD to estimate the LOD and adding 10 times the SD to estimate the LOQ.

(c) *Repeatability and intermediate reproducibility*.—Repeatability (r) and intermediate reproducibility (iR) were assessed by analyzing samples (containing fructans) in duplicate on at least 6 different days. Excel, or the in-house statistical package Q-Stat, were used to calculate the SD(r) and SD(iR) using the following equations:

$$SD(r) = \sqrt{\frac{\sum_{i=1}^{n} SD_i^2}{n}} = \sqrt{\frac{\sum_{i=1}^{n} (x_{i1} - x_{i2})^2}{2n}}$$

$$SD(iR) = \sqrt{SD^2(b) + \frac{1}{2} \times SD^2(r)}$$

where n = the number of (single or duplicate) determinations; x_i = the individual result within the set of single determinations, with i going from 1 to n; x_{i1} and x_{i2} = the two results within the set of a duplicate determination, with i going from 1 to n; and $SD(b)$ = the SD between the means of duplicates.

(d) *Recovery*.—Recovery was assessed slightly differently in the two different laboratories. At NRC, several different infant formulas (containing no fructans) were spiked with three different levels of three different fructan ingredients (Table **2016.14H**). The fructan content of the ingredients was separately determined following Method **997.08** (3). The spiked samples were then analyzed in duplicate on 3 different days, and the recovery was calculated by comparing the measured amount with the theoretical (expected) amount. At CCC, six samples (containing fructans) were spiked with an additional 50 or 150% of the native fructan content (using the same three different fructan ingredients; Table **2016.14I**). The samples were also analyzed in duplicate on 3 different days, and the recoveries were calculated by comparing the theoretical spike amount with the measured spike amount.

Results

Method Development

The method essentially consists of three stages: (*1*) removal of sucrose and free sugars, (*2*) hydrolysis of fructan to release glucose and fructose, and (*3*) analysis of the released glucose and fructose by HPAEC–PAD.

To optimize all parameters, the final HPAEC–PAD method was first developed. In this case, the two laboratories developed different approaches: NRC used a CarboPac PA20 column, and CCC used a CarboPac PA1 column (representative chromatograms are shown in Figure 1). Each system has a dedicated elution gradient, as described in **J**. In both cases, the glucose and fructose are well separated from other sugars, including galactose, which may be released from lactose if the inulinase used for fructan hydrolysis is insufficiently specific. The appearance of galactose in the chromatogram can thus be used as an indicator for this side activity. Both laboratories added sodium hydroxide solution postcolumn, before PAD. The postcolumn addition of sodium hydroxide results in improved baseline stability and higher detector sensitivity. The amperometric detector has a thin-layer flow cell. Due to the impedance in the amperometric flow cell and the resulting ohmic drop in the potential of the working electrode, calibration curves of amperometric detectors deviate from linearity, especially at higher analyte concentrations (9); therefore, both laboratories used quadratic calibration models.

Table 2016.14H. Design of spike-recovery experiment at NRC

Sample No.	Sample description	Pure fructan ingredient			
		Level 0[a]	Level 1[b]	Level 2[c]	Level 3[d]
15	Infant Formula Powder, Milk-Based	None	Orafti P95	Orafti HP	NutraFlora P-95
16	Infant Formula Powder, Soy-Based	None	Orafti HP	NutraFlora P-95	Orafti P95
18	Adult Nutritional RTF, High-Protein	None	NutraFlora P-95	Orafti P95	Orafti HP
11	Adult Nutritional Powder, Low-Fat	None	Orafti P95	Orafti HP	NutraFlora P-95
7	Infant Formula Powder, Partially Hydrolyzed Milk-Based	None	Orafti HP	NutraFlora P-95	Orafti P95
13	Infant Elemental Powder	None	NutraFlora P-95	Orafti P95	Orafti HP

[a] Level 0 = 0 g/100 g.

[b] Level 1 = 0.03 g/100 g.

[c] Level 2 = 2 g/100 g.

[d] Level 3 = 5.0 g/100 g.

Table 2016.14I. Design of spike-recovery experiment at CCC

Sample No.	Sample description	Spike level	Day 1: Orafti P95 spike, g/100 g	Day 2: NutraFlora P-95 spike, g/100 g	Day 3: Orafti HP spike, g/100 g
1	Child Formula Powder	Low	0.17	0.17	0.19
		High	0.49	0.50	0.53
9	Toddler Formula Powder, Milk-Based	Low	0.17	0.17	0.19
		High	0.49	0.50	0.53
10	Infant Formula Powder, Milk-Based	Low	0.17	0.17	0.19
		High	0.49	0.50	0.53
12	Child Formula Powder	Low	0.17	0.17	0.19
		High	0.49	0.50	0.53
14	Infant Formula Powder, FOS/GOS-Based	Low	0.017	0.017	0.019
		High	0.049	0.050	0.053
19	Adult Nutritional RTF, High-Fat	Low	0.17	0.18	0.19
		High	0.49	0.50	0.53

The removal of sucrose is a particularly important part of the method; if not removed, it will erroneously be included in the final fructan concentration. Sucrose can effectively and specifically be hydrolyzed using a sucrase, as described in Method **999.03** (4). However, after hydrolysis, instead of applying a sodium borohydride reduction to remove the released monosaccharides, we have used SPE on a graphitized carbon column. The starting conditions for the SPE were taken from the method described by Cuany et al. (8); however, using the conditions described, it was noted that monosaccharides were not always 100% removed from some products. This problem was investigated and it was found that when the sugars themselves were applied (or the hydrolysate of pure sucrose), all sugars were removed. We concluded that in certain matrixes, there was a component of the sample retained in the SPE column, which in turn was retaining the monosaccharides (in particular, glucose). To overcome this, a wash with sodium chloride solution was introduced. In most cases, this was sufficient to disrupt the interaction, and the monosaccharides were sufficiently removed. However, in a few instances, small amounts of glucose were still retained, even after the sodium chloride wash. The amount retained is very low and, therefore, only significantly impacts the result when very low levels of fructan are being analyzed. To address this issue, we introduced the blank subtraction. To generate the blank, the sample is taken through the whole procedure but not treated with inulinase. Thus, any erroneously trapped monosaccharides can be measured, and the apparent fructan content of the blank can be subtracted from the result of the normally processed sample in order to achieve an accurate result.

The fructan hydrolysis employs the same enzymes as used in Method **999.03** (4). However, the sample is eluted from the SPE in a mixture of acetonitrile and dilute TFA, which is not an optimal condition for inulinase function. Previously (8), the samples were vacuum-dried after SPE to remove the organic solvent and the TFA. However, vacuum-drying adds a considerable amount of time to the analysis. Therefore, we investigated whether the enzymes could function in the presence of acetonitrile after pH adjustment, which was found to be the case. Thus, after SPE, all that is required is the addition of sufficient buffer to adjust the pH, and then the enzymes function as normal. The amount of enzyme added was adapted to ensure complete hydrolysis of all fructans up to a content of 100% in powder products.

Despite regular communication between the two laboratories, the SLV was executed in each laboratory using slightly different protocols (Figure 2). However, the basic principle and major steps of the method remain the same.

Lack-of-Fit Calibration

For both HPAEC–PAD systems (using the CarboPac PA 20 column and the CarboPac PA1 column), good quadratic calibrations for both fructose and glucose were obtained, with extended dynamic ranges and low relative residuals calculated from the differences in the predicted concentration and the actual concentration of the standards (Figure 3). The generally accepted criteria for a good calibration model is that the lack-of-fit for the standards should be less than 5%, with the exception of the lowest standard. It is accepted that the lack-of-fit of one

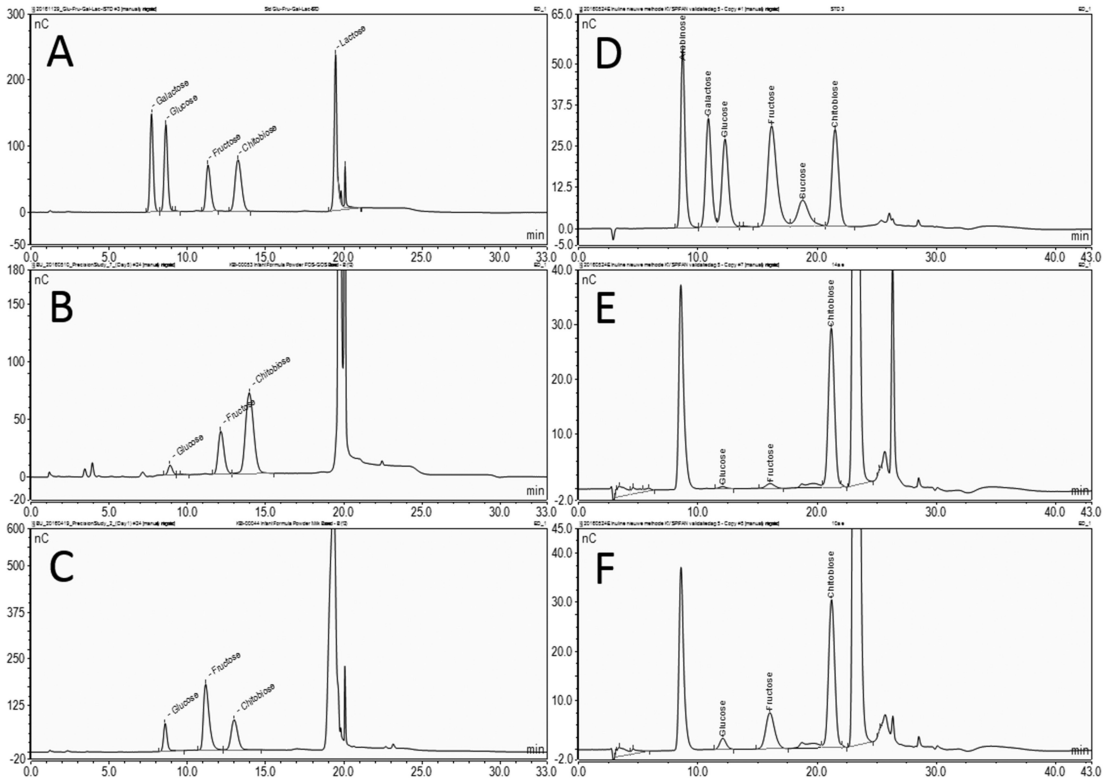

Figure 1. Representative chromatograms of (A) standards separated on the PA20 column; formula containing a fructan concentration of (B) around 0.03 g/100 g and (C) around 0.28 g/100 g on the PA20 column; (D) standards separated on the PA1 column; and formula containing a fructan concentration of (E) around 0.03 g/100 g and (F) around 0.28 g/100 g on the PA1 column.

of the lowest standards may be higher. In both laboratories and for both analytes, the residuals at all but the lowest level are less than 5%, and at the lowest level, they are below 10%.

LOD and LOQ

NRC established the LOD and LOQ by analyzing the blank sample Infant Formula Powder, Soy-Based, Lot No. E10NWZC (Table **2016.14A**, sample No. 16) 14 times (7 days in duplicate). Minor signals present in the sample were quantified as fructan, and the average fructan content (on a reconstituted basis) was determined to be 0.0025 g/100 g with an SD of 0.0016 g/100 g. The LOD and LOQ were estimated using the following equations, and the results meet the requirements in the SMPRs (5).

$$\mathrm{LOD} = 0.0025 + \left(3 \times 0.0016\right) = 0.0073\,\mathrm{g}/100\mathrm{g}$$

$$\mathrm{LOQ} = 0.0025 + \left(10 \times 0.0016\right) = 0.018\,\mathrm{g}/100\mathrm{g}$$

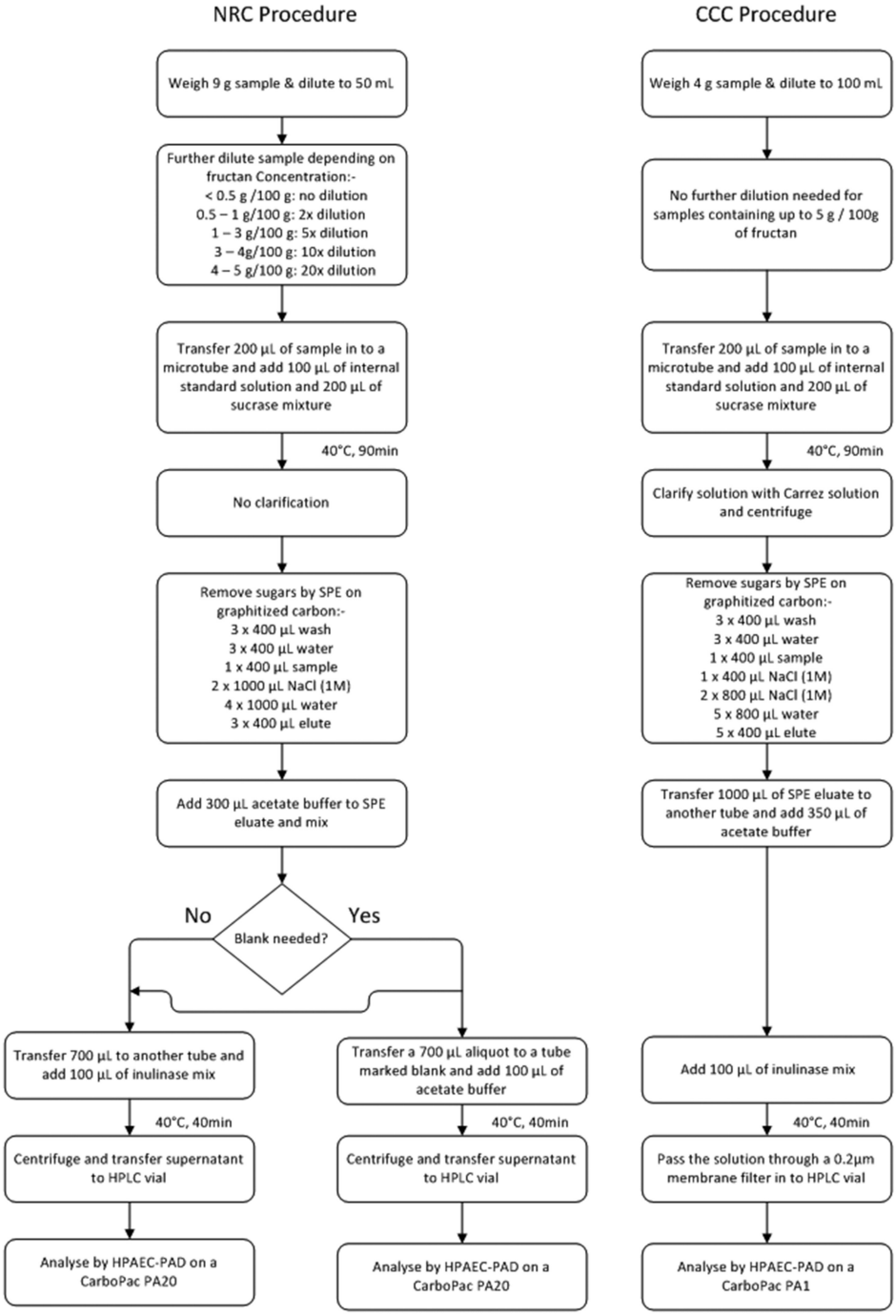

Figure 2. Comparison of the method protocols followed by the two different laboratories during the SLV.

CCC used the sample Infant Formula Powder, Partially Hydrolyzed Milk-Based, Lot No. 410057652Z (Table **2016.14A**, sample No. 7) for the determination of the LOD and LOQ by spiking it with a low level of fructan (Orafti P95, 0.046 g/100 g) and analyzing the sample 10 times on 2 different days.

The LOD and LOQ were then calculated as

$$\text{LOD} = 3 \times \text{SD}$$

$$\text{LOQ} = 10 \times \text{SD}$$

The LOD and LOQ results are summarized in Table 1 and also meet the requirements outlined in the SMPRs (5). The established values of the LOD and LOQ of both NRC and CCC are in good agreement and meet the SMPRs (5).

Precision

It was known that only a few samples in the SPIFAN sample kit contained fructan. To establish which samples contained fructan, both NRC and CCC analyzed the whole series of 19 samples independently from each other. In both laboratories, six of the 19 samples were found to contain fructans:

(1) Sample No. 1, Child Formula Powder, Lot No. 00847RF00.

(*2*) Sample No. 9, Toddler Formula Powder, Milk-Based, Lot No. 4052755861.

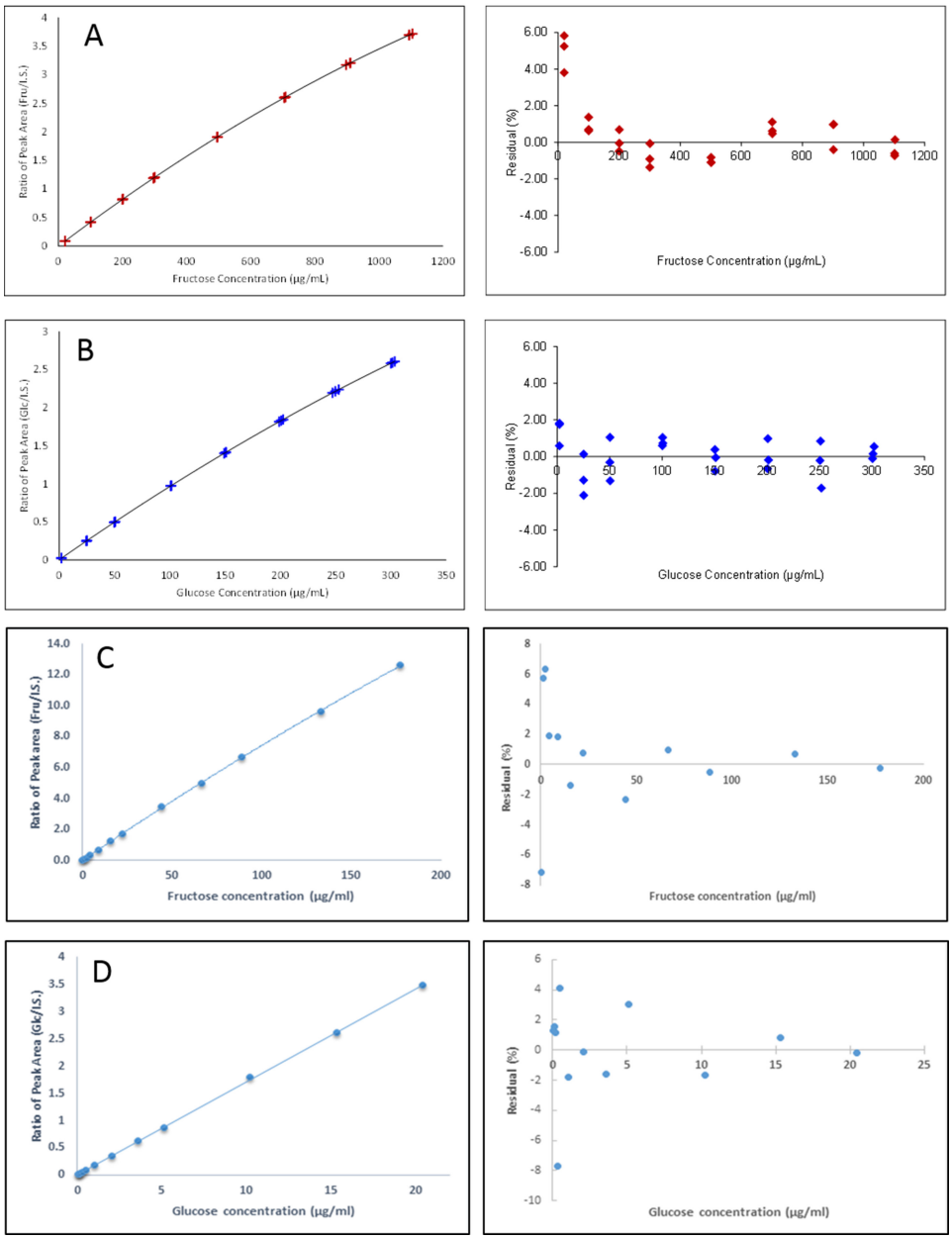

Figure 3. Calibration curves using chitobiose as internal standard (I.S.; left column) and plots of the relative residuals (right column). (A) Fructose (Fru) at NRC; (B) glucose (Glc) at NRC; (C) fructose at CCC; and (D) glucose at CCC.

Table 1. Determination of LOD and LOQ at CCC

Parameter	Day 1[a]		Day 2[a]	
	Fructan content, g/100 g	Meets the SMPRs	Fructan content, g/100 g	Meets the SMPRs
Average[b]	0.044	NA[c]	0.048	NA
SD[b]	0.002	NA	0.003	NA
LOD[d]	0.005	Yes	0.009	Yes
LOQ[e]	0.016	Yes	0.030	Yes

[a] Days 1 and 2 were separated by 3 months.

[b] Average and SD values were calculated on 10 replicates measured on a single day.

[c] NA = Not Applicable.

[d] LOQ calculated as 10SD.

[e] LOD calculated as 3SD.

(*3*) Sample No. 10, Infant Formula Powder, Milk-Based, Lot No. 4044755861.

(*4*) Sample No. 12, Child Formula Powder, Lot No. 00866RF00.

(*5*) Sample No. 14, Infant Formula Powder, FOS/GOS-Based, Lot No. 50350017W1.

(*6*) Sample No. 19, Adult Nutritional RTF, High-Fat, Lot No. 0729RF00.

These six samples were used in the precision study in both laboratories, with an additional two samples (Nestlé Ref 1 and 2) included at NRC. The repeatability and intermediate reproducibility were assessed by analyzing all samples in duplicate on 6 different days, with the exception of Nestlé Ref 2, which was analyzed in duplicate on 24 different days (Table 2).

The repeatability (RSD_r) achieved in both laboratories (Table 2) is well below the upper level of 6% defined in SMPR 2014.002 (5), with only one exception, sample No. 14, which had an RSD_r of 8.9% at CCC. The results for sample No. 14 may be expected to be more variable because this sample contains the lowest fructan content, about 0.03 g/100 g, which is close to the LOQ. The intermediate reproducibility (RSD_{iR}) achieved in both laboratories is, in general, below 8%, again with the exception of sample No. 14 analyzed at CCC. Sample No. 14 was analyzed with an RSD_{iR} of 14%, which is almost certainly linked to the low concentration of analyte in that sample. No limits were defined for RSD_{iR} in SMPR 2014.002 (5), but the data suggest that achieving an RSD_R of below 12% (as defined in SMPR 2014.002) for a multilaboratory trial could be possible, although it may be expected that samples containing fructan levels close to the LOQ may have higher variability.

The analytical results and the established precision data of NRC and CCC agree very well with each other. There is no significant difference in the mean fructan content of the samples [paired *t*-test ($\alpha = 0.05$)], but the RSD_{iR} values of CCC are slightly higher than those of NRC (Table 2). This could be explained by the fact that CCC was able to introduce more variability in the execution of the intermediate reproducibility experiments than NRC: data at CCC were collected over the course of 4 months, whereas at NRC, the data were mostly collected over the course of 1 month; and data at CCC were acquired by two different people using three different instruments, whereas at NRC, most of the precision data were generated by a single person using two different instruments.

Accuracy/Trueness

Primary indications of the accuracy of the method were obtained at CCC during the determination of LOD and LOQ, because this was performed by spiking low levels of fructans into a blank matrix (Table 1). The fructan addition rate was 0.046 g/100 g on both days; thus, the average recoveries were 95.6% on day 1 and 104% on day 2, which are well within the defined target range of 90–110%.

Table 2. Summary of results from the precision study

Sample No.	Sample description	*n*[a]		Mean fructan content, g/100 g		RSD_r, %		Meets SMPR 2014.002 target		RSD_{iR}, %	
		NRC	CCC	NRC	CCC	NRC	CCC	NRC	CCC	NRC	CCC
1	Child Formula Powder	6 × 2	6 × 2	0.27	0.29[b]	0.8	1.4[b]	Yes	Yes[b]	1.1	7.8[b]
9	Toddler Formula Powder, Milk-Based	6 × 2	6 × 2	0. 21	0.22	2.0	1.6	Yes	Yes	2.4	6.1
10	Infant Formula Powder, Milk-Based	6 × 2	6 × 2	0.28	0.26	2.4	1.2	Yes	Yes	2.8	6.9
12	Child Formula Powder	6 × 2	6 × 2	0.28	0.28	1.2	1.1	Yes	Yes	1.1	6.1
14	Infant Formula Powder, FOS/GOS-Based	6 × 2	6 × 2	0.03	0.04	0.9	8.9[c]	Yes	No[c]	2.5	14.2[c]
19	Adult Nutritional RTF, High-Fat	6 × 2	6 × 2	0.48	0.51	1.8	2.6	Yes	Yes	1.6	4.2
Nestlé Ref 1	Infant formula	6 × 2	NA[d]	2.9	NA	1.1	NA	Yes	NA	1.8	NA
Nestlé Ref 2	Infant formula	24 × 2	NA	0.39	NA	3.3	NA	Yes	NA	4.3	NA

[a] *n* = Number of determinations, represented as days × replicates.

[b] There was one (Grubbs) outlier in one of the duplicates; therefore, the results are based on a set of five duplicates instead of six.

[c] The fructan content was close to the LOQ, resulting in somewhat increased RSD_r and RSD_{iR} values.

[d] NA = Not Applicable.

For the full spike-recovery experiments, three different pure fructan ingredients were used: Orafti HP, Orafti P95, and NutraFlora P-95. The ingredients were separately analyzed using Method **997.08** (3) to confirm their purity.

At NRC, six different blank matrixes were spiked at three levels with the above-mentioned three pure fructan ingredients on 3 different days in duplicate. All samples were initially analyzed without using the blank subtraction (Table 3).

At the two higher spiking levels, recoveries were, in general, very good (92–104%), with one exception—the Adult Nutritional RTF, High-Protein sample—for which the average recovery was only 86% at the highest spike level (0.03 g/100 g), which is equivalent to the LOQ specified in the SMPRs (5), the recoveries were less good, varying from 101 to 151%, with three matrixes achieving the SMPRs (recoveries of 101–105%) and three matrixes being outside the requirements (recoveries of 114–151%). Because the spike level is very low, a small amount of interference can have a significant impact on the recovery. To correct for this interference, the method using the blank subtraction was applied. Using the blank subtraction, recoveries on the samples with low spike levels are significantly improved to 95–119% (Table 4) but still do not meet the SMPRs in all cases [recoveries for two matrixes exceeded 110% (i.e., sample No. 7 at 117% and sample No. 18 at 119%)]. This improvement demonstrates the need for the blank subtraction for some samples, especially those containing low levels of fructans.

At CCC, the six fructan-containing samples were overspiked at about 50 and 150% levels of the original fructan content determined in the precision study. All samples, both nonspiked and spiked, were analyzed without using the blank subtraction. The average recoveries (Table 5) were all within the target range of 90–110% defined in SMPR 2014.002 (5), with the exception of one sample (sample No. 9), which had an average recovery of 89% at the low spike level.

Most of the spike-recovery data give acceptable results despite the fact that the method contains an inherent issue that can lead to underestimation of fructan content for some ingredient types. The issue lies in the calculation in which all the fructose is multiplied by a factor of 0.9 to correct for water uptake during hydrolysis. For fructan chains containing a terminal glucose (GFn type), this is not a problem because the glucose is not corrected and 100% recovery can always theoretically be achieved. However, for fructan chains that do not contain a terminal glucose (Fm type), there will be a small underestimation of fructan depending on the chain length [i.e., the degree of polymerization (DP)]. Thus, the theoretically achievable recovery (due to calculation alone) is less than 100% for many fructan ingredients, depending on the average DP and the GFn-to-Fm ratio (Table 6). The worst case is a fructan ingredient containing 100% Fm-type chains and having an average DP of 3, for which only a 96% recovery is achievable; however, in practice, no such ingredient exists. The most impacted ingredient that we are aware of would be a fructan ingredient with an average DP of around 4 and an Fm-to-GFn ratio of 5. Such a product has a theoretically possible recovery of 97.7%. We believe that this small theoretical underestimation should not be a major issue in most cases and has not had a major impact in this study. However, when the laboratory knows the average DP of the fructan ingredient being used, the calculation can be adapted to avoid the underestimation as follows:

$$C_G = C_{GB} \times D \times \left(V/m\right) \times 0.0001$$

$$C_F = C_{FB} \times D \times \left(V/m\right) \times 0.0001$$

$$TF = \left(C_F + C_G\right) \times \left(\left(\left(DP\text{-}1\right) \times 0.9 + 1\right) \Big/ DP\right)$$

where C_G = the concentration (g/100 g) of glucose released from fructan; C_{GB} = the concentration (µg/mL) of glucose in Solution B; D = the dilution factor between Solution A and Solution B (from Table **2016.14C**); V = the total volume (mL) of Solution A; m = the amount (g) of sample weighed to prepare Solution A; 0.0001 = the factor to convert analyte concentration (µg/mL) in solution to analyte concentration (g/100 g) in sample; C_F = the concentration (g/100 g) of fructose released from fructan; C_{FB} = the concentration (µg/mL) of fructose in Solution B; TF = the total fructan concentration (g/100 g) in the sample; 0.9 = the factor to correct for uptake of water during fructan hydrolysis; and DP = the average DP of the fructan ingredient.

Method Specificity

There are potentially two different mechanisms that may cause interference in the method: (*1*) an interfering substance could coelute with the glucose or fructose, and (*2*) the presence

Table 3. Spike-recovery results at NRC

		Level 1			Level 2			Level 3		
Sample No.	Sample description	Spike, g/100 g	Recovery, %	RSD, %	Spike, g/100 g	Recovery, %	RSD, %	Spike, g/100 g	Recovery, %	RSD, %
7	Infant Formula Powder, Partially Hydrolyzed Milk-Based	0.031	122	7.3	2.00	103	2.5	5.01	92.0	2.2
11	Adult Nutritional Powder, Low-Fat	0.031	102	5.1	1.99	102	2.0	5.02	102	1.6
13	Infant Elemental Powder	0.030	105	5.2	2.02	95.7	1.8	5.00	95.5	6.1
15	Infant Formula Powder, Milk-Based	0.031	101	5.0	2.00	99.7	2.2	5.02	98.2	2.4
16	Infant Formula Powder, Soy-Based	0.030	114	3.0	2.02	104	4.2	5.02	93.6	2.8
18	Adult Nutritional RTF, High-Protein	0.030	151	11	1.99	95.5	2.2	4.95	86.0	3.8

Table 4. Spike-recoveries when blank subtraction was applied for the low spike level at NRC

Sample No.	Sample description	Spike, g/100 g	Recovery, %	RSD, %
7	Infant Formula Powder, Partially Hydrolyzed Milk-Based	0.031	117	12
11	Adult Nutritional Powder, Low-Fat	0.031	95.6	7.4
13	Infant Elemental Powder	0.030	96.0	5.6
15	Infant Formula Powder, Milk-Based	0.031	95.4	2.6
16	Infant Formula Powder, Soy-Based	0.030	104	11
18	Adult Nutritional RTF, High-Protein	0.030	119[a]	7.1

[a] Sample analyzed on 4 days in duplicate; all other samples analyzed on 3 days in duplicate.

of glucose- and/or fructose-containing carbohydrates (oligo- and/or polysaccharides), which are susceptible to hydrolysis by (side) activities of the fructanase used in the last enzymatic hydrolysis step in the protocol. Both mechanisms would lead to overestimation of the fructan content.

Table 5. Spike-recovery results at CCC

Sample No.	Sample description	Addition spiked level (g/100 g)	Average recovery, %	SD_{Rec}
1	Child Formula Powder	Low (0.17–0.19)	90.8	6.3
		High (0.49–0.53)	95.7	3.8
9	Toddler Formula Powder, Milk-Based	Low (0.17–0.191)	89.0	4.7
		High (0.49–0.53)	93.2	1.2
10	Infant Formula Powder, Milk-Based	Low (0.17–0.19)	94.1	2.1
		High (0.49–0.53)	94.6	2.9
12	Child Formula Powder	Low (0.17–0.19)	91.0	4.9
		High (0.49–0.53)	101.5	7.1
14	Infant Formula Powder, FOS/GOS-Based	Low (0.017–0.019)[a]	92.8[b]	6.3
		High (0.049–0.053)	92.2	5.5
19	Adult Nutritional RTF, High-Fat	Low (0.17–0.19)	94.8	9.2
		High (0.49–0.53)	95.3	4.5
Average recovery			93.7	

[a] Spiked level is less than the LOQ concentration of 0.03 g/100g.

[b] One (Grubbs) outlier recovery value (56.8%) was rejected.

Table 6. Impact of calculation on theoretical recovery

Ratio of fructan types		Average DP								
GFn	Fm	3	4	5	6	8	10	20	50	100
		Theoretical recovery due to calculation, %								
1	0	100	100	100	100	100	100	100	100	100
1	1	98.2	98.6	98.9	99.1	99.3	99.5	99.7	99.9	99.9
1	2	97.6	98.2	98.6	98.8	99.1	99.3	99.6	99.9	99.9
1	3	97.3	98.0	98.4	98.6	99.0	99.2	99.6	99.8	99.9
1	4	97.1	97.8	98.3	98.5	98.9	99.1	99.6	99.8	99.9
1	5	97.0	97.7	98.2	98.5	98.9	99.1	99.5	99.8	99.9
1	10	96.8	97.5	98.0	98.3	98.8	99.0	99.5	99.8	99.9
1	50	96.5	97.4	97.9	98.2	98.7	98.9	99.5	99.8	99.9
0	1	96.4	97.3	97.8	98.2	98.6	98.9	99.4	99.8	99.9

The specificity of the method is achieved through a combination of the specificity of the enzymes used for the sample preparation and the selectivity of the chromatographic system used for the final analysis. To confirm the method had sufficient specificity, a number of pure carbohydrate constituents that may be present in infant formula and adult nutritionals were subjected to the analysis (following the CCC procedure) in order to determine whether or not they falsely contribute to the fructan content. The following carbohydrates were tested: resistant maltodextrin, soluble starch, isomaltulose, maltitol, sucrose, galactooligosaccharides (GOS), and polydextrose, all with 0.5 g sample weight. The results (Table 7) have been expressed as if the carbohydrate represented 12.5% of a reconstituted (or RTF) sample and 50% of a dry powder. The results (Table 7) have also been recorded with and without inclusion of the blank subtraction step.

The analytical results summarized in the column "Ingredient as 12.5% in RTF" show clearly that after applying the standard protocol without blank correction, none of the potentially interfering constituents, with the exception of polydextrose, gave rise to an erroneously high fructan content. All of the measured fructan levels were shown to be significantly lower than the LOQ. For polydextrose, the erroneously measured fructan content is near the level of the LOQ. However, when the blank correction is applied, the interference is consistently below 0.01 g/100 g. The chomatographic profiles of polydextrose and

Table 7. Results of specificity experiments

	Fructan content, g/100 g			
	Ingredient as 12.5% in RTF		Ingredient as 100% of dry product	
Ingredient	No blank correction	With blank correction	No blank correction	With blank correction
Resistant dextrin	0.004	0.002	0.035	0.014
Soluble starch	0.010	0.009	0.077	0.069
Isomaltulose	0.005	0.004	0.037	0.033
Maltitol	0.002	0.001	0.015	0.007
Sucrose	0.006	0.005	0.049	0.041
GOS	0.023	0.002	0.182	0.017
Polydextrose	0.034	0.007	0.271	0.058

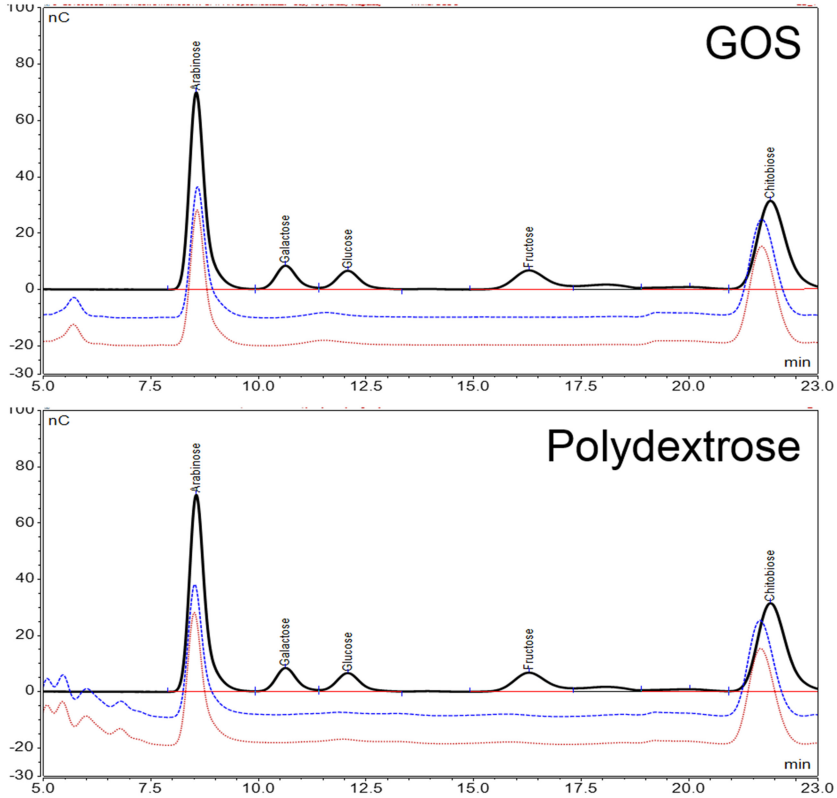

Figure 4. The dotted line designates the chromatogram with no blank correction; the dashed line designates the chromatogram with blank correction; the solid line indicates the standards arabinose, galactose, glucose, fructose and chitobiose.

GOS on the CarboPac PA1 column (Figure 4) contains signals near the fructose and glucose peaks. Although the retention times differ somewhat from the calibration standards, it is likely that they could interfere if present at very high concentrations; however, at typical usage levels in adult nutritionals and infant formula, they should not represent a problem.

In the column "Ingredient as 100% of dry product" with no blank correction (Table 7), GOS and polydextrose resulted in the highest erroneous fructan content (approximately 0.2–0.3 g/100 g); the other ingredients produced results below 0.1 g/100 g. Applying the blank correction resulted in a significant improvement, and all ingredients produced results below 0.1 g/100 g. These data indicate that those ingredients would have a negligible influence on the analysis of fructans in actual products.

Conclusions

The performance of this new method, as established by two independent laboratories, largely meets the requirements outlined in SMPR 2014.002 (5), and the specificity and selectivity of the method are good. The good agreement of results between the two laboratories also indicates that the method is sufficiently robust to resist the minor changes in protocols between the two laboratories. The reduced number of chromatographic runs and the elimination of the need for ingredient-specific correction factors should be a significant advantage over the previous AOAC *Official Methods*^{SM} **997.08** (3) and **999.03** (4)] for the determination of the total fructan content in formula and adult nutritionals.

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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.

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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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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.

Table 1. Summary of SLV acceptance criteria from SMPR 2011.009 and SMPR 2014.004

	SMPR 2014.004									SMPR 2011.009		
Parameter	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

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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.

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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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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

(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 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

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(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

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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.

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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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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.

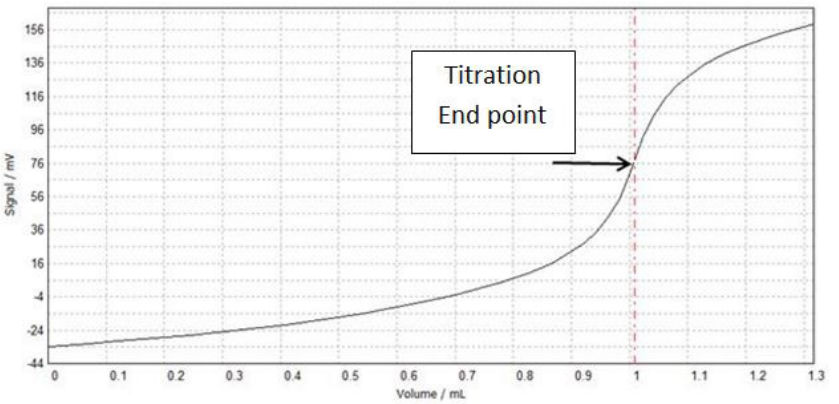
(e) *NaCl solution, 0.1 M.*—If no ready-to-use NaCl standard solution is available, weigh 5.8440 ± 0.0005 g NaCl, previously dried for 2 h at 110 ± 2°C. Dissolve in water and make up to the mark in a 1000 mL volumetric flask.

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

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(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

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

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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.
[1] Corresponding author's e-mail: karen.schimpf@abbott.com
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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.

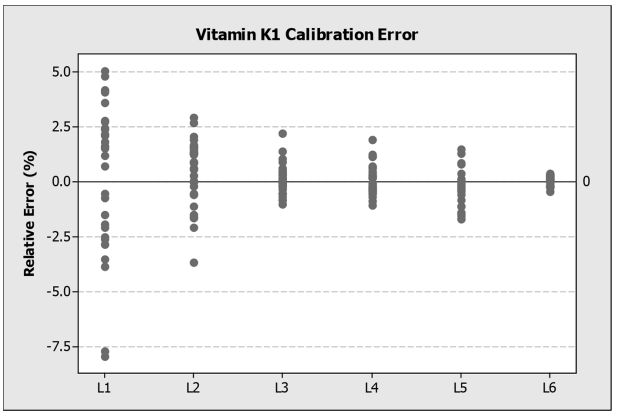

Figure 1. Vitamin K_1 standard curve calibration errors; L = level.

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.

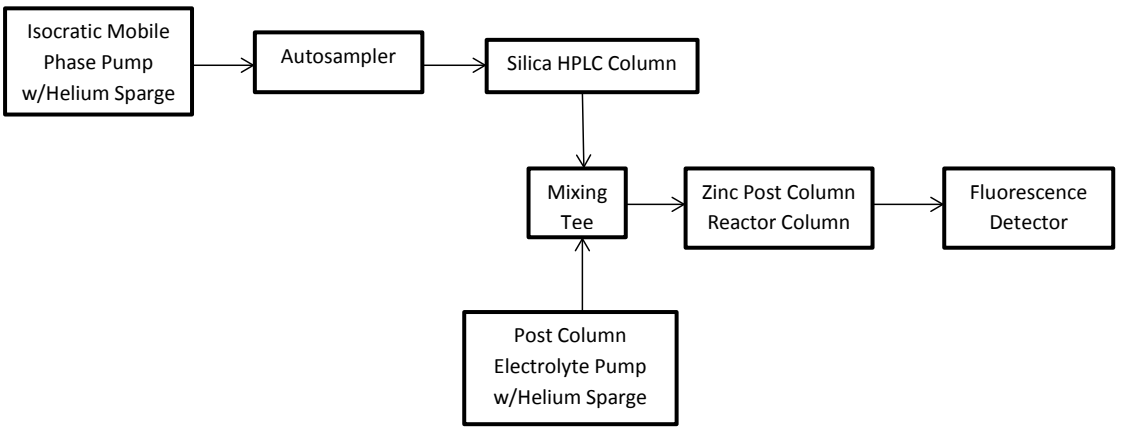

Figure 2015.09A. Vitamin K system configuration.

(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

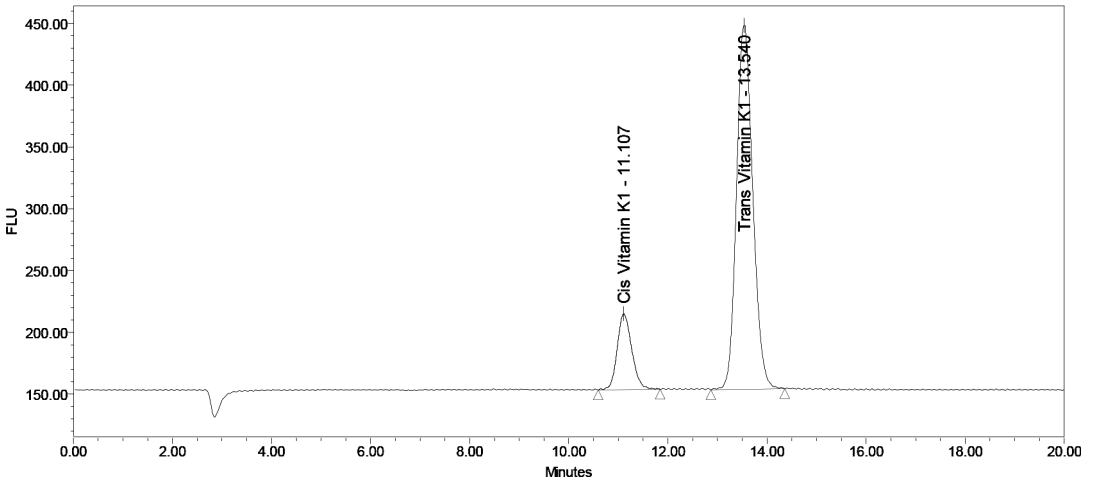

Figure 2015.09B. Chromatogram of vitamin K_1 standard.

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

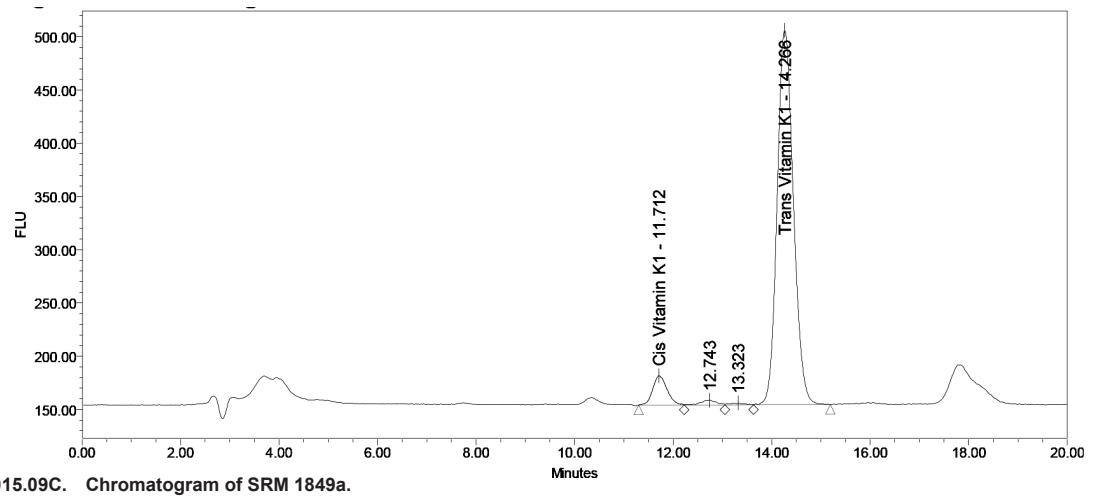

Figure 2015.09C. Chromatogram of SRM 1849a.

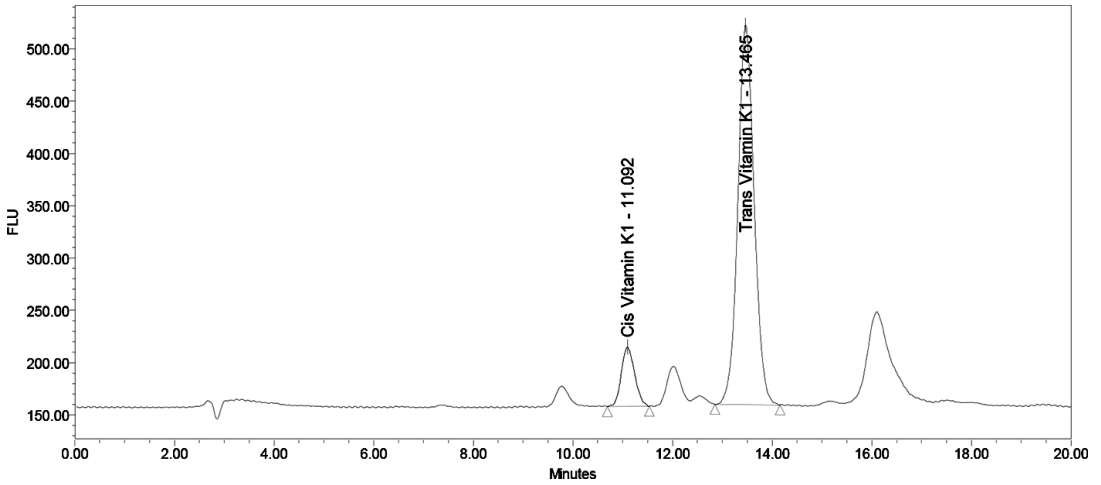

Figure 2015.09D. Chromatogram of an adult nutritional.

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.

References

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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.
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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

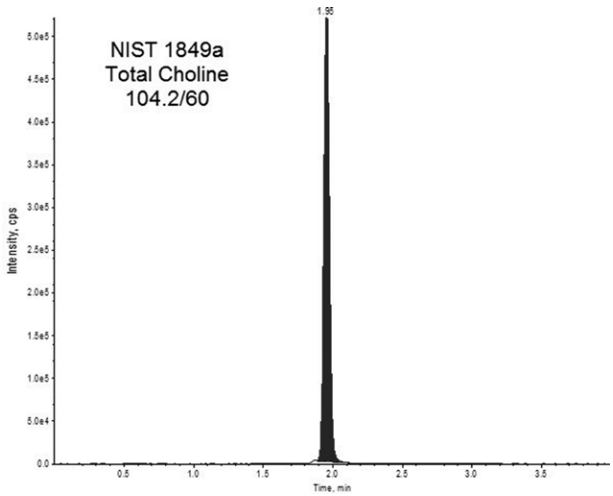

Figure 2015.10A Extracted ion chromatogram (XIC) of choline.

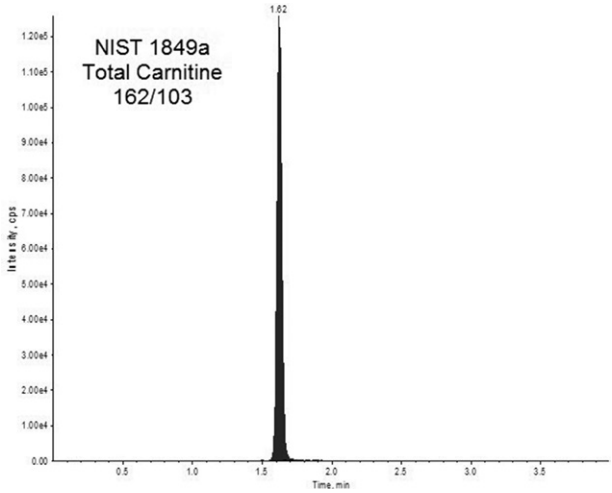

Figure 2015.10B. Extracted ion chromatogram (XIC) of carnitine.

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.

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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

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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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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:

$$\left[Vit\right]_{Stk} = \frac{W_s \times M \times S \times P \times 1{,}000}{Vol}$$

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.

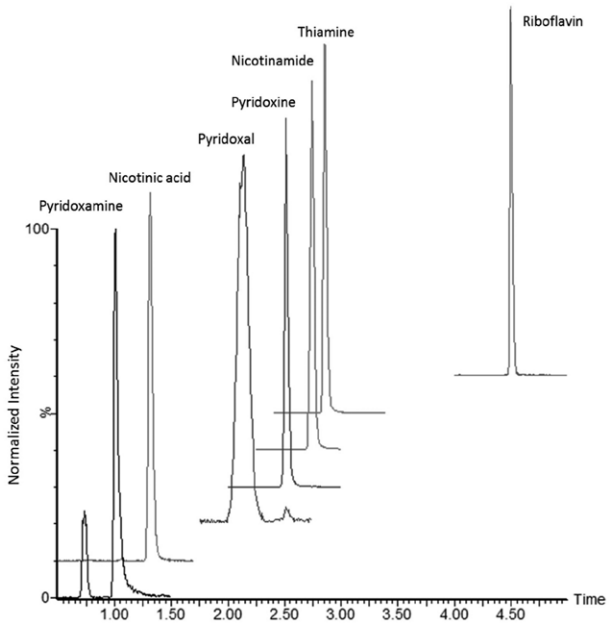

Figure 1. A chromatogram for the seven vitamin forms in the child formula powder. The data are unsmoothed, and the intensity of each peak is normalized to aid visualization. There is more than two orders of magnitude difference in signal intensity, which makes the small features such as pyridoxamine 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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INFANT FORMULA AND ADULT NUTRITIONALS

Improved AOAC First Action 2011.08 for the Analysis of Vitamin B_{12} in Infant Formula and Adult/Pediatric Formulas: First Action 2014.02

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This report documents improvement and single-laboratory validation performed on AOAC First Action Method 2011.08 for vitamin B_{12} in infant formula and adult/pediatric nutritional formula. The original validation study included a range of fortified products, from infant formulas to breakfast cereals or beverages. Extended validation data, including additional infant formulas and adult/pediatric nutritionals, has now been produced. In addition, the method has been modified to use ultra-HPLC and the calibration range extended in a multilevel calibration curve. Detection and quantification limits were also improved by increasing the sample weight used for analysis and the reconstitution rate adapted to the requirements. The Stakeholder Panel on Infant Formula and Adult Nutritionals Test Material Kit, designed to represent a large range of products within the category (infant formula and adult nutritionals made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein), was used to determine performance characteristics of the method. The modifications included allow now full compliance with standard method performance requirements established for vitamin B_{12} (SMPR 2011.005). LOQ was ≤0.01 µg/100 g, working range between 0.01 and 5.0 µg/100 g, repeatability ≤7%, and recovery in the range 90–110%. The method was granted AOAC First Action status 2014.02.

Based on the data presented in the single-laboratory validation study (SLV) reported by Campos Giménez et al. (1), the method "Determination of Vitamin B_{12} in Infant Formulas and Adult Nutritionals by LC-UV Detection with Immunoaffinity Extraction" was granted First Action status and designated AOAC **2011.08** (2, 3).

The original validation study included a large range of fortified products, not only infant formulas but also breakfast cereals and beverages. The data provided for infant formulas and adult/pediatric nutritionals was limited and needed to be extended to the full set of Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) matrixes. The method has been further improved by introduction of rapid ultra-HPLC (UHPLC), a multilevel external calibration curve, and change of reconstitution rate and sample weight used to comply with SPIFAN requirements. Additional sample preparation for the analysis of amino acid-based products has now been included. These modifications allow full compliance with standard method performance requirements (SMPR) established for vitamin B_{12} (4) in terms of LOQ (≤0.01 µg/100 g), working range (0.01–5.0 µg/100 g), repeatability (≤7%) and recovery (90–110%). The improved method was granted AOAC First Action status **2014.02**.

AOAC Official Method 2014.02
Vitamin B_{12}
in Infant Formula and Adult/Pediatric Formulas
Ultra-High-Performance Liquid Chromatography
First Action 2014

[Applicable for the determination of vitamin B_{12} in all forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates), made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein.]

Caution: The method uses commonly used solvents and reagents. Refer to appropriate manuals or safety data sheets to ensure that the safety guidelines are applied before using chemicals.

Cyanide.—Fatal if swallowed, inhaled, or comes in contact with skin. Wear protective gloves, clothing, and eyewear. Wash hands immediately after handling the product. Cyanide reacts with acids to form highly toxic and rapid acting HCN gas. Use only in effective fume removal device to remove vapors generated. Destroy residues with alkaline NaOCl solution.

Trifluoroacetic acid (TFA).—Causes severe burns and eye damage. Wear protective gloves, clothing, eyewear, and face protection. Use only in effective fume removal device to remove vapors generated.

See Table **2014.02A** for samples used during validation of the method. The set is composed of six nonfortified (placebo) products and 12 fortified products. It also includes a Standard Reference Material, SRM 1849a Infant/Adult Nutritional

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The method was approved by the Expert Review Panel for Infant Formula as First Action.
The Expert Review Panel for Infant Formula 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 at http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
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Table 2014.02A. SPIFAN SLV test materials kit information

	Product description
Fortified products	Infant formula powder, partially hydrolyzed, milk-based
	Infant formula powder, partially hydrolyzed, soy-based
	Infant elemental powder
	Infant formula powder, milk-based
	Infant formula powder, soy-based
	Infant formula ready-to-feed, milk-based
	Child formula powder
	Adult nutritional powder, milk protein-based
	Adult nutritional powder, low-fat
	Adult nutritional ready-to-feed, high-protein
	Adult nutritional ready-to-feed, high-fat
	SRM 1849a Infant/Adult Nutritional Formula
Nonfortified (placebo)	Infant elemental powder
	Infant formula ready-to-feed, milk-based
	Child formula powder
	Adult nutritional ready-to-feed, high-protein
	Adult nutritional ready-to-feed, high-fat

Formula, from the National Institute of Standards and Technology (Gaithersburg, MD) with a reference value for vitamin B_{12}.

A. Principle

Vitamin B_{12} is extracted from the sample using sodium acetate buffer in the presence of sodium cyanide at 100°C for 30 min. Extracts are purified and concentrated with an immunoaffinity column. Vitamin B_{12} is determined by ultra-high-performance liquid chromatography with UV detection at 361 nm.

B. Apparatus and Materials

(**a**) *Balances*.—With readability of 0.1 mg (AT200; Mettler-Toledo Inc., Greifensee, Switzerland) and 0.01 g (PE400; Mettler-Toledo Inc.).

(**b**) *Sonicator*.—Bioblock (Fisher Scientific, Wohlen, Switzerland).

(**c**) *Laboratory oven*.—Heraeus (Hanau, Germany), or water bath.

(**d**) *In-line water bath (with magnetic stirrers) or autoclave*.

(**e**) *pH meter*.—Metrohm 691 (Herisau, Switzerland).

(**f**) *Rotary shaker for biochemistry*.—Labnet International (Edison, NJ) or Stuart LB3 (Barloworld, Bibby Sterilin Ltd, Staffordshire, UK).

(**g**) *Heating block*.—With nitrogen evaporation (Pierce Biotechnology, Inc., Rockford, IL).

(**h**) *Vortex*.—Scientific Industries, Inc. (Bohemia, NY).

(**i**) *Homogenizer*.—Polytron PT3000 (drive unit), Aggregate PT-DA 3012 (Kinematica, Lucerne, Switzerland).

(**j**) *Volumetric flasks*.—Amber glass, 10, 50, 100, 200, 250 mL; clear glass, 2000 mL.

(**k**) *Graduated cylinders*.—50, 100, and 1000 mL.

(**l**) *Beakers*.—Amber glass, 250 mL.

(**m**) *Flat-bottom round flasks or Erlenmeyers*.—Amber glass, 250 mL.

(**n**) *Folded paper filters*.—602 1/2 or 597 1/2 (Whatman Inc., Maidstone, UK).

(**o**) *Amber vials*.—Screw top, 7 or 4 mL (Supelco Inc., Bellefonte, PA).

(**p**) *Micro LC vials*.—Amber (Supelco Inc.).

(**q**) *Pipets*.—Graduated glass, 10 mL, or volumetric glass, 9 mL.

(**r**) *Electronic digital pipet*.—Variable volume, 10–100 µL.

(**s**) *Syringes*.—Disposable, 20 mL, equipped with a perforated rubber stopper attached to the tip.

(**t**) *Immunoaffinity columns*.—EASY-EXTRACT VITAMIN B_{12} LGE (R-Biopharm AG, Darmstadt, Germany, www.r-biopharm.com; Product Code P88).

(**u**) *Immunoaffinity column rack*.—Product Code CR1 (R-Biopharm AG).

(**v**) *Chromatographic system*.—Waters Acquity UPLC® including Binary Solvent Manager, Sample Manager, and UV detector (Waters, Milford, MA) or ultra-high-performance chromatography system of equivalent characteristics.

(**w**) *Chromatographic column*.—Waters Acquity UPLC® BEH C18, 1.7 µm, 2.1 × 100 mm (Waters).

C. Chemicals and Solvents

(**a**) *Methanol*.—HPLC grade (Merck, Darmstadt, Germany).

(**b**) *Acetonitrile*.—HPLC grade (Merck).

(**c**) *Acetic acid, glacial*.—Merck.

(**d**) *Milli-Q water*.—Millipore (Bedford, MA). Use throughout where water is specified.

(**e**) *Sodium cyanide puriss*.—Fluka (Buchs, Switzerland).

(**f**) *TFA for spectroscopy*.—Merck.

(**g**) *Vitamin B_{12} (cyanocobalamin), approximately 99%*.—Sigma-Aldrich (St. Louis, MO).

(**h**) *Sodium acetate trihydrate p.a.*—Merck.

(**i**) *Sodium hypochloride*.—Technical grade.

(**j**) *α-Amylase from Bacillus subtilis*.—Approximately 50 units/mg (Sigma-Aldrich); optional.

D. Preparation of Reagents and Standard Solutions

(**a**) *Sodium acetate solution 0.4 M, pH 4.0*.—Into a 2000 mL volumetric flask, weigh 108.8 g sodium acetate trihydrate. Add about 1800 mL water. Dissolve. Add 50 mL acetic acid, and adjust pH to 4.0 with acetic acid. Dilute to volume with water.

(**b**) *Sodium cyanide solution, 1% (w/v)*.—Weigh 0.5 g sodium cyanide into a 50 mL amber glass volumetric flask. Dilute to volume with water. Any excess of 1% sodium cyanide solution must be destroyed by adding 1.5 mL of a 15% solution of sodium hypochlorite per 1 mL sodium cyanide solution. Let react for 2 days in a fume hood. (*Caution:* Sodium cyanide is highly toxic. Avoid contact with skin, and work in a fume hood. Disposal of any unused solutions should comply with local regulations.)

(**c**) *Mobile phase A*.—To 1000 mL water, add 250 µL TFA. Mix well.

(**d**) *Mobile phase B*.—To 1000 mL acetonitrile, add 250 µL TFA. Mix well.

(**e**) *Sample dilution solvent*.—Mix 90 mL mobile phase A with 10 mL mobile phase B.

(**f**) *Vitamin B_{12} stock standard solution (100 µg/mL)*.—Accurately weigh 20.0 mg vitamin B_{12} into a 200 mL amber glass volumetric flask. Add about 150 mL water. Dissolve by sonication and stirring for a few minutes. Dilute to volume with water. Solution is stable for ≥6 months at –20°C. (*Note*: Vitamin B_{12} is sensitive to light. Conduct operations under subdued light, or use amber glassware. Keep all solutions away from direct light.)

(**g**) *Vitamin B_{12} intermediate standard solution (400 ng/mL)*.—Pipet 1 mL vitamin B_{12} stock standard solution into a 250 mL amber glass volumetric flask. Make up to volume with water.

(**h**) *Vitamin B_{12} working standard solutions for calibration (2, 10, 20, 40, 60, 100 ng/mL)*.—Pipet into six separate 10 mL amber glass volumetric flasks, 50, 250, 500, 1000, 1500, and 2500 µL vitamin B_{12} intermediate standard solution. Dilute to volume with sample dilution solvent, (**e**).

E. Sample Preparation and Extraction

(**a**) *Sample reconstitution for powder samples*.—Weigh 25.0 g sample into a 250 mL beaker. Add 200 g water at 40 ± 5°C. Mix with a glass rod until the suspension is homogeneous, or homogenize with a Polytron. Proceed as described in **E**(**d**) *Extraction*.

(**b**) *Sample reconstitution for amino acid-based products*.—Weigh 25.0 g powder sample into a 250 mL beaker. Add 190 g water at 40 ± 5°C and 10 g skimmed milk powder. Mix with a glass rod until the suspension is homogeneous, or homogenize with a Polytron. In parallel, run a blank by replacing the sample by water. Dilute both, reconstituted sample and blank, twice in water (e.g., 50 g reconstituted sample or blank + 50 g water). Proceed as described in **E**(**d**) *Extraction.*

(**c**) *Sample preparation for liquid samples*.—Mix well to ensure homogeneity of the sample portion. Proceed as described in **E**(**d**) *Extraction*.

(**d**) *Extraction*.—Weigh 60.0 g sample suspension, **E**(**a**) and (**b**), blank, **E**(**b**), or liquid sample, **E**(**c**), into a 250 mL flat-bottom amber glass flask or Erlenmeyer with ground glass neck. Add 1 mL of 1% sodium cyanide solution, **D**(**b**). If the sample contains starch, add about 0.05 g α-amylase, mix thoroughly, stopper the flask, and incubate 15 min at 40 ± 5°C. Add 25 mL sodium acetate solution, **D**(**a**). Mix well. Place the flask in a boiling water bath for 30 min (or autoclave 30 min at 100°C). Cool the flask in an ice bath. Quantitatively transfer the content of flask to a 100 mL amber glass volumetric flask. Dilute to volume with water. Filter the solution through a folded paper filter. In the case of high-fat products, and if recovery is low, dilute the filtrate 1:3 in water before cleanup to improve recovery or repeat the extraction by using a smaller sample portion.

(**e**) *Immunoaffinity cleanup*.—Let the immunoaffinity columns warm to room temperature by removing them from refrigeration at least 30 min before use. Place each immunoaffinity column on the rack. Open the caps and let the storage buffer drain by gravity. Close the lower cap. Load the column with 9 mL clear filtrate and close the upper cap. Place the column in a rotary shaker, and mix slowly for 10–15 min. Return the column to the support and let stand for a few minutes. Open the caps to let the liquid drain by gravity. Wash the column with 10 mL water. With a syringe, insert about 40 mL air to dry the column. Elute with 3 mL methanol, and collect eluate in a 4 or 7 mL amber glass reaction vial. Rinse the column with 0.5 mL methanol, and with a syringe, insert about 20 mL air to collect all the methanol in the same vial. Evaporate the eluate at 50°C under a stream of nitrogen. Reconstitute the sample in 0.3 mL sample dilution solvent, **D**(**e**). Mix on a Vortex mixer. Transfer to a micro amber vial.

F. Analysis

(**a**) *Chromatographic conditions*.—Flow rate, 0.4 mL/min; injection volume, 50 µL; detection, UV at 361 nm; gradient elution, *see* Table **2014.02B**.

(**b**) *System suitability test*.—Equilibrate the chromatographic system for at least 15 min. Inject a working standard solution three to six times, and check peak retention times and responses. Inject working standard solutions on a regular basis within a series of analyses. The coefficient of variation should not be higher than 2%.

(**c**) *Analysis*.—Make single injections of standard and test solutions. Measure chromatographic peak response (height).

(**d**) *Identification*.—Identify vitamin B_{12} peak in the chromatograms of the test solution by comparison with the retention time and UV spectrum of the corresponding peak obtained for the standard solution.

(**e**) *Calibration*.—Plot peak responses against concentrations (in ng/mL). Perform regression analysis. Calculate slope and intercept. Check the linearity of the calibration ($R^2 > 0.99$; standard error of calibration < 10%).

(**f**) *Quantitation*.—Calculate the concentration of vitamin B_{12}, in µg/100 g of product as reconstituted, as follows:

$$\frac{(A-I)\times V_0\times V_2\times 100}{S\times m\times V_1\times 1000}$$

where A = response (height) of the peak obtained for the sample solution, I = intercept of the calibration curve, S = slope of the calibration curve, V_0 = volume of the test solution (volume used to dissolve the test portion) in mL (100 mL), V_2 = volume in which the aliquot of sample solution is reconstituted after immunoaffinity cleanup (0.3 mL), m = weight of the test portion, as reconstituted, in g (60 g), and V_1 = volume of the aliquot of sample solution loaded onto the affinity column (9 mL). For amino acid-based products calculate the vitamin B_{12} content on the sample and on the blank, **E**(**e**); take into account the additional dilution factor 1/5 in the calculations. Deduct the amount of vitamin B_{12} in the blank to the amount in the sample.

Table 2014.02B. Gradient elution

Time, min	Mobile phase A, %	Mobile phase B, %
0.0	90	10
1.7	90	10
2.5	75	25
2.9	10	90
3.9	10	90
4.0	90	10
8.0	90	10

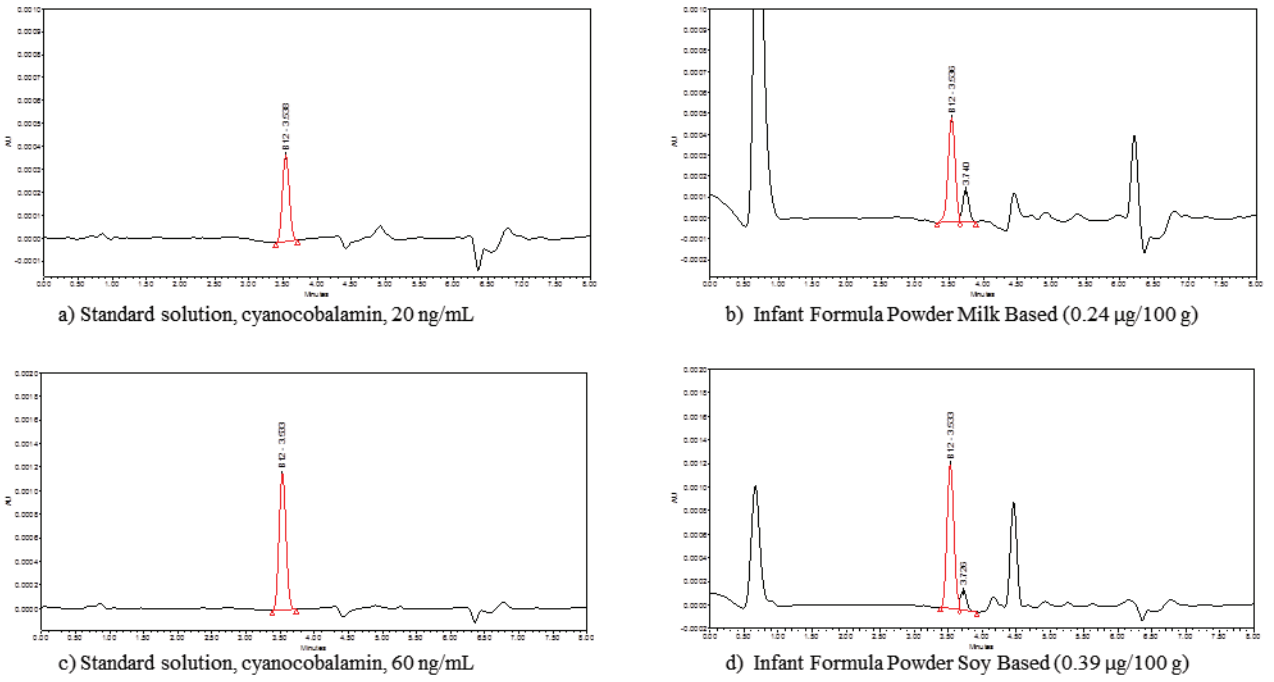


Figure 1. Example chromatograms of standard solutions at 20 (a) and 60 ng/mL (c) and infant formula powders (b, d).

(**g**) *Reporting.*—Report results with two decimal points as cyanocobalamin, in µg/100 g of reconstituted product. Reconstitution rates are 25 g/225 g for powder products, 50 g/100 g for concentrates, and 1 g/1 g for ready-to-feed formulas.

Validation Protocol

(**a**) *Linearity.*—Three independent stock solutions of cyanocobalamin were prepared at a concentration of 100 µg/mL. Working solutions at different levels prepared from dilution of stock solutions were injected in triplicate.

(**b**) *LOD/LOQ.*—Ten independent analysis of a nonfortified liquid sample, overdiluted to obtain a final concentration of about 0.005 µg/100 g, were used for determination of LOD and LOQ as LOD = blank mean + 3 SD and LOQ = blank mean + 10 SD.

(**c**) *Trueness.*—Reference material (SRM 1849a) was analyzed in duplicate over 6 days by two different analysts. Overall mean was calculated and compared to the reference value.

(**d**) *Recovery.*—Spiking experiments were performed at 50 and 100% of typical target levels in infant formula, on three selected nonfortified products. Spiked and nonspiked samples were analyzed in duplicate on 3 different days by two different analysts. The rest of the nonfortified products were spiked and analyzed in duplicate on a single day. The overall mean of unspiked samples was used to compute recoveries.

(**e**) *Precision studies.*—Six fortified samples, including SRM 1849a, were selected for precision studies and analyzed in duplicate on 6 different days by two analysts. Fresh reagents and working standards were prepared each day. Repeatability was verified on the rest of the samples by analyzing them in duplicate on a single day; this was due to insufficient amount of sample available to run on multiple days.

(**f**) *Statistics.*—SD of repeatability (S_r) and SD of intermediate reproducibility (S_{iR}) were used as measures of within-day and between-day variability, respectively. They were calculated from the data obtained in the precision studies as:

$$S_r=\sqrt{\frac{\sum_{i=1}^{n}(x_{i1}-x_{i2})^2}{2n}} \text{ and } S_{iR}=\sqrt{SD^2(b)+\frac{1}{2}Sr^2}$$

where n is the number of duplicate determinations; x_{i1} and x_{i2} are the two single results with i going from 1 to n and $SD^2(b)$ is the SD between the means of duplicates. Recovery rates (%) were calculated from spiking experiments as:

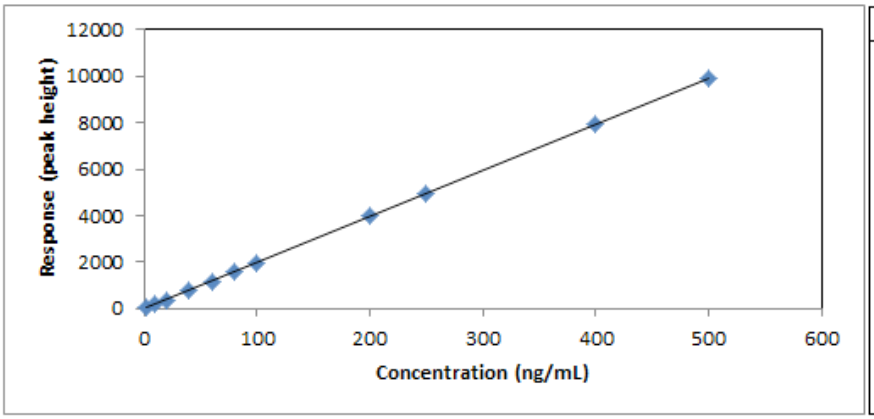
$$\text{Recovery (\%)} = \frac{C_{spiked}-C_{native}}{C_{added}} \times 100$$

where C_{spiked} is the concentration measured in the spiked sample; C_{native} is the concentration measured in the nonfortified sample (overall mean of unspiked samples); and C_{added} is the concentration of analyte added.

Validation Results

Chromatography.—Example chromatograms using the newly validated conditions (UHPLC) are shown in Figure 1. Chromatographic time has been reduced from the previously reported 16 min to about 8 min.

Linearity.—An extended calibration range (from 2 to 500 ng/mL) was used for linearity demonstration (Figure 2). Calibration curves were plotted and linearity demonstrated by $R^2 > 0.9999$ and calibration errors well below 5% for all levels



ng/mL	Response	% Error
2	36	8.6%
10	193	0.7%
20	388	0.5%
40	784	0.3%
60	1189	0.4%
80	1576	0.3%
100	1980	0.1%
200	3967	0.1%
250	4956	0.0%
400	7904	0.4%
500	9939	0.2%

Figure 2. Multilevel calibration curve example including calibration error estimates.

except the lowest concentration (2 ng/mL; corresponding to lower LOQ 0.01 µg/100 g), which showed, in some cases, calibration errors 10–20%. It was considered acceptable at this low level.

During routine analysis, a reduced calibration range from 2 to 100 ng/mL, which covers the range 0.01–0.55 µg/100 g, is recommended. This range can be extended as needed.

LOD/LOQ.—Due to the absence of a matrix devoid of vitamin B_{12} in the SPIFAN kit to be used in establishing LOD and LOQ, a nonfortified product over-diluted to contain about 0.005 µg/100 g was used. The results from 10 independent analyses showed an average of 0.006 µg/100 g, with SD of 0.0007 µg/100 g. Thus, LOD was estimated at 0.008 µg/100 g and LOQ at 0.013 µg/100 g.

Trueness.—Results on SRM 1849a (Infant/Adult Nutritional Formula) are shown in Table 1. The overall mean of duplicate analysis was 0.435 µg/100 g, with $SD_{(b)}$ (SD of the mean of duplicates) of 0.010 µg/100 g, which is well within the reference range of 0.482 ± 0.085 µg/100 g.

Recovery.—Results of spiking experiments are shown in Table 2. Most recoveries obtained using the method as previously described complied with requirements (90–100%), except for the Adult Nutritional ready-to-feed (RTF) High Fat and Infant Elemental Powder, with recoveries around 80% (data not shown).

For those two samples, sample preparation was adapted to allow better recovery rates. Briefly, the Adult Nutritional RTF High Fat was diluted three times in water to reduce matrix effect before extraction; while in the case of the amino acid-based (elemental) product, a source of intact protein (skimmed milk powder) was added to mimic regular matrixes. These adaptations allowed obtaining recovery rates within acceptable ranges. After adaptation, recovery rates in all samples ranged from 87.8 to 98.3%. Mean recovery was 91.7 ± 4.0% (mean ± SD).

Precision.—Precision data are shown in Tables 1 and 3. RSD of repeatability, S_r, was below 7%, except for Infant Formula Powder (S_r = 8.2%) and RSD of intermediate reproducibility, S_{iR}, was not higher than 11%. Repeatability was confirmed on the rest of the matrixes (fortified or not) by duplicate analysis on a single day. Only the Child Formula Powder (nonfortified) showed differences between duplicates higher than 7%.

Conclusions

The adaptations provided to the method allow meeting all requirements specified in the SMPR. Response was linear in the range 2–500 ng/mL, which corresponds to 0.01–2.8 µg/100 g (as reconstituted product); this range can easily be extended by dilution of sample extracts. LOD and LOQ were 0.008 and 0.013 µg/100 g, respectively. Accuracy of the method was proven by successful analysis of a Certified Reference Material (SRM 1849a Infant/Adult Nutritional Formula), as well as by recovery rates generally within 90–110% at 50 and 100% target values for infant formulas. Precision estimations (S_r and S_{iR}) determined in the range 0.2–1.2 µg/100 g were below 7 and 11%, respectively, for all matrixes tested (six selected products) except for Infant Formula Powder Milk Based (S_r = 8.2%).

Table 1. Precision data for infant formula and adult/pediatric formulas[a]

	Mean, *n* = 12	$SD_{(b)}$	S_r	CV_r, %	S_{iR}	CV_{iR}, %
Infant formula powder, partially hydrolyzed, milk-based	0.35	0.019	0.012	3.4	0.021	3.5
Infant formula powder, partially hydrolyzed, soy-based	0.26	0.074	0.007	2.7	0.009	3.3
Infant formula powder, milk-based	0.24	0.017	0.020	8.2	0.022	9.0
Infant formula powder, soy-based	0.43	0.031	0.013	3.0	0.032	7.4
Adult nutritional RTF, high-protein	1.18	0.046	0.042	3.6	0.055	4.6
SRM1849a Infant/Adult Nutritional Formula	0.435	0.010	0.019	4.4	0.017	3.8

[a] All results reported in µg/100 g of reconstituted product (reconstitution rate 25 g + 200 g water) or ready-to-feed. Mean of duplicate analysis performed by two different analysts on 6 different days. $SD_{(b)}$ = SD of mean of duplicates; S_r = SD of repeatability; CV_r = RSD of repeatability; S_{iR} = SD of intermediate reproducibility; CV_{iR} = RSD of intermediate reproducibility.

Table 2. Recovery results in nonfortified samples[a]

			Level 1		Level 2	
	n	Native content	Recovery, %	CV, %	Recovery, %	CV, %
Child formula powder	6	0.10	96.8	4.3	98.3	3.5
Adult nutritional RTF, high-protein	2	0.03	89.7	4.7	89.8	2.4
Infant formula RTF, milk-based	6	0.05	92.6	4.9	93.3	7.1
Adult nutritional RTF, high-fat	6	0.04	87.8	3.1	87.8	3.0
Infant elemental powder	6	0.00	90.2	3.5	91.1	3.0

[a] *n* = Number of days. Levels 1 and 2 are 0.15 and 0.30 µg/100 g for all products except infant elemental powder, for which level 1 is 2.25 µg/100 g and Level 2 is 4.50 µg/100 g. Native content is reported in µg/100 g of reconstituted or RTF product (reconstitution rate 25 g + 225 g water).

Table 3. Precision verification for infant formula and adult/pediatric formulas[a]

	Mean, *n* = 2	SD, %
Adult nutritional powder, milk protein-based	0.31	3.9
Adult nutritional powder, low-fat	0.67	1.3
Child formula powder	0.94	1.9
Infant elemental powder	0.60	0.5
Adult nutritional RTF, high-fat[b]	1.40	12.2
Infant formula RTF, milk-based	0.32	6.3
Child formula powder (nonfortified)	0.10	11.5
Adult nutritional RTF, high-protein (nonfortified)	0.03	3.9
Adult nutritional RTF, high-fat (nonfortified)[b]	0.04	5.6

[a] Mean of duplicate analysis on a single day. All results reported in µg/100 g of reconstituted product (reconstitution rate 25 g + 200 g water) or RTF.

[b] Results obtained without further dilution of sample previous to extraction.

The method was found suitable for the determination of vitamin B_{12}, in the form of cyanocobalamin, as well as the naturally occurring forms (mainly hydroxyl-, adenosyl-, and methylcobalamin) in infant formula and adult/pediatric formula.

References

(1) Campos Giménez, E., Fontannaz, P., Trisconi, M.J., Kilinc, T., Gimenez, C., & Andrieux, P. (2008) *J. AOAC Int.* **91**, 786–793

(2) Campos Giménez, E., Fontannaz, P., Trisconi, M.J., Kilinc, T., Gimenez, C., & Andrieux, P. (2012) *J. AOAC Int.* **95**, 307–312. http://dx.doi.org/10.5740/jaoacint.CS201108

(3) *Official Methods of Analysis* (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2011.08**

(4) AOAC SMPR **2011.005** (2012) *J. AOAC Int*. **95**, 293. http://dx.doi.org/10.5740/jaoacint.11-0441

INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Free and Total Carnitine and Choline in Infant Formulas and Adult Nutritional Products by UPLC/MS/MS: Single-Laboratory Validation, First Action 2014.04

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A single-laboratory validation (SLV) has been performed for a method that simultaneously determines choline and carnitine in nutritional products by ultra performance LC (UPLC)/MS/MS. All 11 matrixes from the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) were tested. Depending on the sample preparation, either the added (free, with a water dilution and filtering) or total (after microwave digestion at 120°C in nitric acid and subsequent neutralization with ammonia) species can be detected. For nonmilk containing products, the total carnitine is almost always equal to the free carnitine. A substantial difference was noted between free and total choline in all products. All *Standard Method Performance Requirements* for carnitine and choline have been met. This report summarizes the material sent to the AOAC Expert Review Panel for SPIFAN nutrient methods for the review of this method, as well as some additional data from an internal validation. The method was granted AOAC First Action status for carnitine in 2014 (2014.04), but the choline data are also being presented. A comparison of choline results to those from other AOAC methods is given.

Over the last 3 years, as part of the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) project, AOAC has issued regular Calls for Methods to find suitable test methods for global dispute resolution purposes for nutritional products. Choline was considered early in the process. *Standard Method Performance Requirement* (SMPR®) **2012.013** was developed and approved in 2012, describing the performance requirements needed for a choline dispute-resolution method. Three methods were chosen for consideration from those submitted. Abbott Nutrition submitted AOAC **2012.19**, a UPLC-MS/MS method similar to the present submission. In September 2012, SMPR **2012.010** was developed and approved for carnitine. Abbott Nutrition submitted AOAC **2012.17**, a carnitine-only LC/MS-based method (1) in response to the Call for Methods, and this was the only carnitine entry for some time.

In 2013, the Expert Review Panel (ERP) clarified that, for a total choline method, it was not necessary to prove recovery of the individual inherent choline species such as phosphocholine and sphingomyelin; therefore, the choline method could be made much simpler and combined with carnitine. Carnitine was added as an analyte, similar to Andrieux et al. (2), but now using MS/MS for added specificity. Fu et al. (3) reported a choline single-laboratory validation (SLV) in infant formula using MS/MS but did not look at simultaneous carnitine measurement. Microwave digestion was added to speed up the typical 3 h digestion, similar to Phillips and Sander (4), but again the present work utilizes MS/MS instead of single quadrupole technology. The combined method was substantially different than either AOAC **2012.19** or **2012.17**, and so both methods were withdrawn from the SPIFAN process. The present submission of AOAC **2014.04** has a full set of SLV data with the method in its final form. It is submitted in response to a second Call for Methods for carnitine, but full SLV data are also presented for choline in case the ERP also wants to advance it for this use.

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The method was approved by the AOAC Expert Review Panel for Infant Formula and Adult Nutritionals as First Action.

The AOAC Expert Review Panel for SPIFAN Nutrient Methods 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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AOAC Official Method 2014.04
Simultaneous Determination of Choline/Carnitine in Infant Formulas and Adult Nutritional Products
HILIC LC/MS/MS
First Action 2014

A. Principle

Reconstituted test sample is weighed into a microwave reaction vessel. Microwave heating accelerates an acidic hydrolysis process to release bound choline. A subsequent alkaline degradation is performed to release L-carnitine from inherent acylcarnitines. Choline and L-carnitine can be determined quantitatively in nutritional products and raw materials by hydrophilic interaction ultra-performance LC with tandem mass spectrometry (HILIC-UPLC/MS/MS).

B. Apparatus and Materials

(**a**) *Column.*—Acquity UPLC ethylene bridged hybrid

Table 2014.04A. Volumes of each stock solution for IWS

IWS	Stock vol., µL	MIX-IS, µL	Final vol., mL
1	25	100	50
2	125	100	50
3	250	100	50

(BEH) HILIC 1.7 µm, 2.1 × 100 mm (Waters Corp., Milford, MA) or equivalent.

(**b**) *Liquid chromatograph.*—Waters Acquity UPLC Binary or equivalent.

(**c**) *Solvent manager.*—Capable of 15 000 psi or equivalent. (Waters Corp.)

(**d**) *Detector.*—Waters Acquity TQD or Xevo TQS triple quadrupole mass spectrometer (Waters Corp.) or equivalent.

(**e**) *Injector.*—Waters Acquity sample manager with integrated column oven or equivalent.

(**f**) *Autosampler*.—Waters Acquity with cooler and vials, or equivalent.

(**g**) *Nitrogen generator*.—Peak Scientific (Billerica, MA) Model NM30LA or equivalent.

(**h**) *Data system.*—Waters MassLynx, latest revision or equivalent.

(**i**) *Microwave digestion system.*—MarsXpress (CEM, Matthews, NC) or equivalent.

(**j**) *Reaction vessel.*—50 mL Teflon® (CEM) or equivalent.

(**k**) *Centrifuge tubes.*—Polypropylene, 50 mL capacity, disposable, or equivalent.

(**l**) *Vortex mixer with flat and cone top.*

(**m**) *Balance 1.*—Readable to at least 0.0001 g (Mettler-Toledo AT200, Columbus, OH) or equivalent.

(**n**) *Balance 2.*—Readable to at least 0.001 mg (Mettler-Toledo XP 6) or equivalent.

(**o**) *Syringe.*—1 mL Luer tip.

(**p**) *Filters.*—0.45 µm nylon membrane with syringe tip or equivalent.

(**q**) *Pipets.*—5–25, 50–250, and 100–1000 µL adjustable.

(**r**) *Pipet tips.*

(**s**) *pH meter.*

(**t**) *Volumetric flasks.*—10, 50, 100, 250 mL.

C. Chemicals and Solvents

(**a**) *Acetonitrile.*—Fisher Optimal (Fisher Scientific, Fair Lawn, NJ) LC/MS grade, or equivalent.

(**b**) *Nitric acid.*—69–70% reagent grade, J.T. Baker (Avantor Center Valley, PA) or equivalent.

(**c**) *Ammonium acetate.*—Fluka (Sigma-Aldrich, St. Louis, MO) LC/MS additive grade or equivalent.

(**d**) *Ammonium hydroxide.*—28–30% reagent grade [for adjusting pH in mobile phase also; check before use; MACRON (Avantor Center Valley, PA) or equivalent)].

(**e**) *Deionized laboratory water.*—≥18 Mohm/cm (EMD Millipore Corp. Billerica, MA, or equivalent).

(**f**) *pH buffer solutions*.—pH 4.0, 7.0, 10.0.

D. Standards

(**a**) *L-Carnitine*.—USP Reference Standard Cat. No. 1359903 or equivalent; store desiccated.

(**b**) *L-Carnitine-d_3 HCl (methyl-d_3)*.—Cambridge Isotope Laboratories Inc. (Andover, MA) DLM-1871-0.1; store refrigerated.

(**c**) *Choline chloride*.—USP Reference Standard Cat. No. 1133547 or equivalent.

(**d**) *Choline chloride-d_9 (trimethyl-d_9 98%)*.—DLM-549-1 (Cambridge Isotope Laboratories Inc.).

E. Preparation of Reagents/Standard Solutions

(**a**) *Mobile phase.*—(*1*) *Mobile phase A.*—10 mM ammonium acetate [water–acetonitrile (95 + 5, v/v)]. Quantitatively transfer 770 (±15) mg ammonium acetate into a 1 L glass bottle and dissolve with 950 mL laboratory water. Adjust pH up to 8.2–8.6 by ammonium hydroxide, **C**(**d**). Record the amount of ammonium hydroxide added. Add 50 mL acetonitrile to the bottle and mix well. Store at room temperature. Expiration: 1 week.

(*2*) *Mobile phase B.*—10 mM ammonium acetate [water–acetonitrile (5 + 95, v/v)]. Weigh 770 (±15) mg ammonium acetate into a 100 mL beaker. Dissolve with 50 mL laboratory water. Add same amount of ammonium hydroxide as added into mobile phase A and then mix well. Quantitatively transfer the 50 mL solution into a 1 L glass bottle. Add 950 mL acetonitrile into the bottle and mix well. Store at room temperature. Expiration: 2 weeks.

(**b**) *Native stock solutions.*—(*1*) *Choline hydroxide (approximately 3000 µg/mL).*—Store at 2–8°C. Expiration: 1 month (currently trying to extend to 1 year). (*a*) Dry approximately 200 mg choline chloride USP Reference Standard

Table 2014.04B. Concentration of standards

	µg/mL					
Standard	Choline	Choline chloride-d_9	Carnitine	Carnitine chloride-d_3	Diluent	Final vol., mL
IWS 1	1.5	1.6	0.375	0.8	Water	50
IWS 2	7.5	1.6	1.875	0.8	Water	50
IWS 3	15	1.6	3.750	0.8	Water	50
	ng/mL				90%	
WS 1	30	32	7.5	16	MeCN[a]	10
WS 2	150	32	37.5	16	MeCN	10
WS 3	300	32	75	16	MeCN	10

[a] MeCN = Acetonitrile.

Table 2014.04C. Microwave digestion parameters

Power	100% (1600 W)
Ramp to temperature, min	10
Hold time, min	40
Temperature, °C	120

in a vacuum oven at 65°C (±3°C) for 4 h. (*b*) Weigh 170 (±17) mg into an appropriate weighing boat. (*c*) Quantitatively transfer into a 50 mL volumetric flask. (*d*) Dissolve and bring to volume with laboratory water. (*Note*: choline chloride is the form weighed, but many laboratories prefer working in units of choline hydroxide, which is how the method is presented here.)

(*2*) *L-Carnitine (approximately 750 µg/mL).*—(*a*) Weigh 150.0 ± 8 mg into an appropriate weighing boat as quickly as possible. (*b*) Transfer into a 200 mL volumetric flask. (*c*) Dissolve and bring to volume with laboratory water. (*d*) Correct weight for moisture content. (*e*) Aliquot the stock solution into 2 mL plastic vials, with each vial receiving at least 1 mL and store ≤–15°C. Expiration: 1 year.

(**c**) *Internal standard (IS) stock solutions.*—(*1*) *Choline chloride-d_9 (approximately 20 000 µg/mL).*—(*a*) Transfer 1 g choline chloride-d_9 into a 50 mL volumetric flask. (*b*) Dissolve and bring to volume with laboratory water. (*c*) Aliquot the stock solution into 2 mL plastic vials with each vial receiving about 1 mL and store at ≤–15°C. Expiration: 1 year.

(*2*) *L-Carnitine chloride-d_3 HCl (approximately 10 000 µg/mL).*—(*a*) Transfer 0.5 g L-carnitine chloride-d_3 to a 50 mL flask. (*b*) Dissolve and bring to volume with laboratory water. (*c*) Aliquot the stock solution into 2 mL plastic vials with each vial receiving about 1 mL and store at ≤–15°C. Expiration: 1 year.

(**d**) *Mixed intermediate internal standard solution (MIX-IS).*—Prepare fresh on day of analysis. Must use same MIX-IS solution for both calibration working standards and samples.

(*1*) Transfer 400 µL of each choline chloride-d_9 and 400 µL carnitine chloride-d_3 HCl stock solution by a calibrated pipet into a 10 mL volumetric flask.

(*2*) Bring to volume with laboratory water and mix well.

(**e**) *Intermediate working standards (IWS).*—Prepare fresh on day of analysis.

(*1*) Pipet 100 µL MIX-IS into three individual 50 mL volumetric flasks.

(*2*) Using Table **2014.04A**, add the required volumes of the stock choline and carnitine solution by proper sized calibrated pipets.

(*3*) Bring to volume with laboratory water and mix well.

(**f**) *Working standard solution (WS).*–Prepare fresh on day of analysis. Use 90% acetonitrile in final working solution.

(*1*) Pipet 200 µL of each IWS (IWS 1–3) into three 10 mL volumetric flasks.

(*2*) Add 800 µL laboratory water to each volumetric flask.

(*3*) Bring to volume with acetonitrile and mix well.

(*4*) Transfer to autosampler vials for analysis.

(*5*) Table **2014.04B** summarizes the concentrations of choline and carnitine and their associated ISs in the three IWS and in the three WS.

F. Procedure

(**a**) *Liquids.*—For liquids and ready-to-feed products, select sample weights between 1 and 5 g based on expected concentration of the two nutrients in each sample.

(**b**) *Powders.*—Powder products are reconstituted with water prior to analysis, typically 11.1% (w/w; 25 g powder added to 200 g water). Typically a 5 g sample aliquot is taken from the reconstituted material for analysis, but adjust as needed.

(**c**) *Sample analysis.*—(*1*) Weigh sample into a tarred microwave reaction vessel.

(*2*) Add 125 µL MIX-IS.

(*3*) Add water to sample to achieve a total sample volume of approximately 8 mL.

(*4*) Add 2.2 mL of approximately 70% nitric acid and seal vessels. The final volume should be close to 10 mL (sample + acid + water + MIX-IS = 10 mL) prior to microwave digestion which makes acid concentration approximately 3.5 M.

(*5*) Vortex vessels for 30 s, and then place vessels into turntable and insert into microwave system.

(*6*) Complete microwave digestion using the conditions defined in Table **2014.04C**. Operate with microwave venting to a fume hood.

(*7*) After cool down (by air), uncap each vessel, and add 3.5 mL concentrated ammonium hydroxide to each vessel. Operate in a fume hood.

(*8*) Vortex each sample for 30 s.

(*9*) Allow samples to stand in a hood for 30 min to react any acylcarnitines under basic conditions.

(*10*) Vortex samples for 30 s.

(*11*) Transfer 1 mL digested sample into a disposable centrifuge tube containing 25 mL laboratory water.

(*12*) Vortex for 30 s.

(*13*) Filter about 1 mL diluted sample using a 0.45 µm nylon syringe filter.

(*14*) Transfer 0.1 mL filtered solution into autosampler vial containing 0.9 mL acetonitrile. Mix well. Sample is ready for analysis.

(*15*) Samples diluted with acetonitrile are stable for 24 h

Table 2014.04D. Mass analysis parameters (Xevo TQ-S)

Standard	Retention time typical, min	Molecular ion (precursor), amu	Product ion, amu	Dwell, s	Cone voltage, V	Collision energy, V
Choline	2.5	104.2	60.3	0.025	40	32
Choline chloride-d_9	2.5	113.4	69.3	0.025	40	30
Carnitine	4.4	162	103	0.025	30	28
Carnitine chloride-d_3	4.4	165	103	0.025	30	26

Table 2014.04E. Mass spectrometer (Xevo TQ-S) operating conditions

Ionization mode	ESI positive
Capillary voltage, kV	2.0
Collision gas pressure, mtorr	2–5 × 10^{-3}
Source temperature, °C	150
Source offset, V	30
Desolvation temperature, °C	550
Cone gas flow, L/h	300
Desolvation flow rate, L/h	1000
Peak width half-height, amu[a]	0.7

[a] Instrumental resolution parameters for the TQ-S are set up by IntelliStart to achieve a resolution of approximately 0.7 amu across the mass range.

Table 2014.04F. Chromatographic parameters

Mobile phase A	10 mM ammonium acetate [water–acetonitrile (95 + 5, v/v)]
Mobile phase B	10 mM ammonium acetate [water–acetonitrile (5 + 95, v/v)]
Flow rate	0.7 mL/min (analytical)
Flow rate into MS	Full flow
Column	Acquity UPLC BEH HILIC 1.7 µm, 2.1 × 100 mm
Column temperature,	25
Injection volume, µL	10
Injection type	Full loop
Sample temperature, °C	8

Table 2014.04G. Gradient profile

		Mobile phase		
Time, min	Flow, mL/min	A, %	B, %	Curve
Initial	0.7	8.0	92.0	
0.10	0.7	8.0	92.0	6
6.00	0.7	22	78	6
6.01	0.7	100	0	6
8.00	0.7	100	0	6
10.00	1.0	8.0	92.0	6
13.00	1.0	8.0	92.0	6
13.20	0.7	8.0	92.0	6
15.00	0.7	8.0	92.0	6

when stored in a refrigerated (8°C) autosampler. Filtered samples (aqueous) and WS are stable for 7 days stored at 2–8°C.

(**d**) The method can also determine free choline and free carnitine by bypassing the microwave digestion and basic hydrolysis. Simply dilute the same sample size with water, add 125 µL of MIX-IS, and dilute to 10 mL in a tube. Mix, and then dilute this solution 1 mL to 25 mL with water in another tube. Mix, filter approximately 1 mL of sample slurry, and then dilute 10x with acetonitrile as above. No nitric acid or ammonia is used.

Table 1. Method performance requirements

	Choline (a)	Carnitine (b)
Analytical range	2–250	0.16–20
LOD	0.7	NA[a]
LOQ	2.0	≤0.16
Repeatability (RSD_r)	10% at 2 mg/100 g; otherwise 5%	≤8%
Recovery	90–110% at all levels over range	90–110% at all levels over range
Reproducibility (RSD_R)	15% at 2 mg/100 g; otherwise 10%	≤15%
	(a) mg/100 g concentrations apply to: (*1*) "ready-to-feed" liquids "as is"; (*2*) reconstituted powders (25 g into 200 g water); and (*3*) liquid concentrates diluted 1:1 by weight. For all concentrations, choline will be expressed as mg/100 g reconstituted liquids. Report as total choline.	(b) mg/100 g concentrations apply to: (*1*) "ready-to-feed" liquids "as is"; (*2*) reconstituted powders (25 g into 200 g water); and (3) liquid concentrates diluted 1:1 by weight.

[a] NA = Not applicable.

Table 2. SLV test materials

Product type	Code	Sample size used for repeatability SLV, g
SRM 1849a	NA[c]	5.0[a]
Infant powder milk	D04HTCVV	4.3[a]
Infant powder soy	E29JVLV	3.7[a]
Infant powder milk part hydrolyzed	1172572116	3.9[a]
Infant powder soy part hydrolyzed	117257651Z	4.1[a]
Adult powder low fat	00394RF00	4.1[a]
Adult powder milk	11750017V3	7.5[a,b]
Child formula powder	00412RF00	2.7[a]
Infant elemental powder	00403RF00	3.7[a]
Infant RTF milk	SPIFAN CTL	3.0
Adult RTF high protein	00414RF00	0.8
Adult RTF high fat	00406RF00	0.6

[a] Aliquot size from an 11.1% (w/w) reconstitution in water (25 g powder plus 200 g water).

[b] An abnormally large sample size was needed for this sample, in which both choline and carnitine were present at <LOQ.

[c] NA = Not applicable.

G. Instrument Operating Conditions

(**a**) *MS conditions.—See* Tables **2014.04D** and **E**.

(**b**) *UPLC conditions.–See* Tables **2014.04F** and **G**.

(**c**) *UPLC analysis*.—Column stability was improved by storing the column in water–acetonitrile (5 + 95, v/v) without

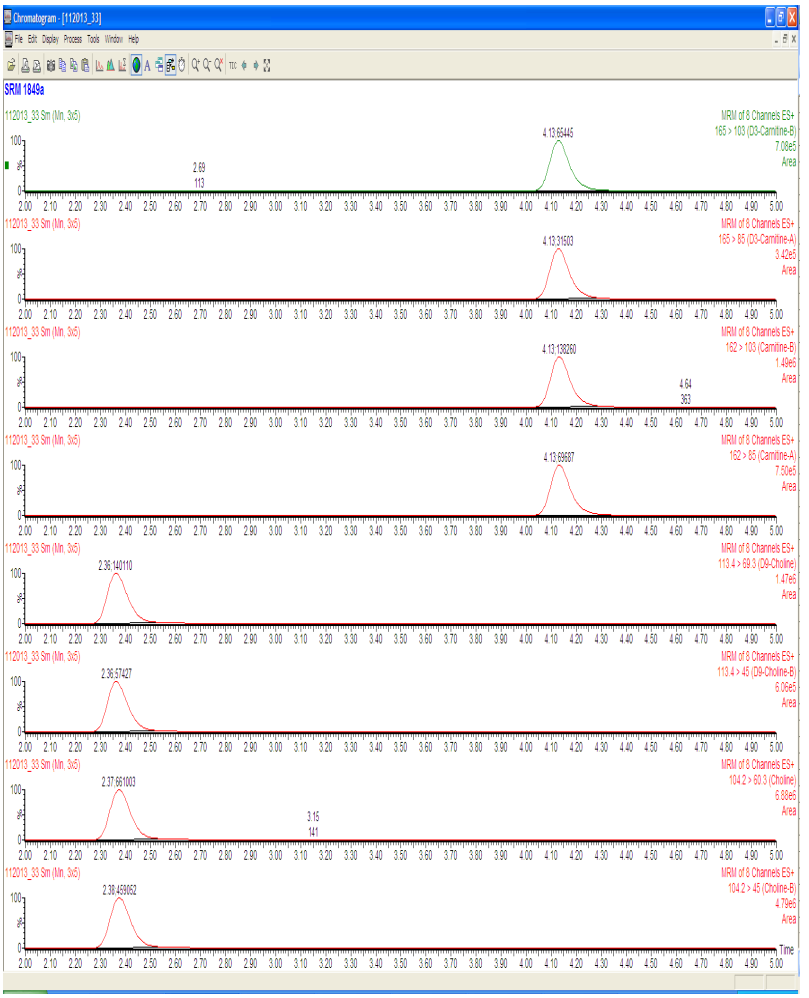

Figure 1. Representative chromatograms of SRM 1849a (total analysis). Secondary transitions were monitored for both choline and carnitine, including their internal standards (only the primary transitions are given in Table 2014.04D).

additives (recommended by column supplier). After verifying equilibration of the UPLC system, inject the mid-level working standard four times to verify system suitability. RSD of the peak areas from these injections should be <5%. Once system suitability has been established, inject working standards (WS 1–3), followed by a reagent blank, control sample, and samples. Reinject working standards approximately every 4 h (e.g., enough time for 16 samples with analysis cycle time of 15 min).

H. System Suitability

(**a**) The RSD of the four standard injections to prove equilibration prior to run must be <5%.

(**b**) Calibration curve residuals must be ≤4%. Samples should be bracketed by two sets of such valid calibration curves.

(**c**) A suitable control sample is National Institute of Standards and Technology Standard Reference Material (NIST SRM) 1849a, reconstituted as a normal sample powder (each packet contains about 10 g). A control sample must be run concurrently with every sample set and a corresponding control chart set up. The control chart RSD of the means of choline and carnitine must be <4.0%.

(**d**) The method is valid for analytical solution concentrations between 50% of WS1 and 10% above WS3.

I. Calculations

(**a**) For each of the three WS, the software plots each relative response (analyte/internal standard) versus its corresponding working standard concentration to obtain two separate calibration curves for choline and carnitine (two data points for each concentration, one from the beginning of the analysis and one from the end). It applies a linear regression model to the data and obtains an equation for the best-fit line.

(**b**) For each sample injected, the instrument measures the response (analyte/internal standard) for choline and carnitine and uses the linear regression equation to calculate the resulting concentration in the analytical solution.

(**c**) The concentration in the analytical solution (ng/mL) is multiplied by a dilution factor (DF) to project the results back to the original sample, on a µg/g basis:

$$C_x = C_s \times DF \quad (1)$$

where C_x is the concentration of the analyte in the product (µg/g) and C_s is the concentration in the analytical solution measured by the instrument (ng/mL).

Table 3. Linearity results[a]

Nominal carnitine, µg/L	Recovery, % (avg. 3 days)	RSD, %
3.4	101.2	1.8
6.9	99.3	1.2
17.2	100.3	1.1
34.5	100.0	1.5
51.7	99.4	0.7
68.9	100.1	1.5
Nominal choline, µg/L	**Recovery, % (avg. 3 days)**	**RSD, %**
29.7	100.3	2.0
59.3	99.6	1.7
119	100.3	2.2
237	99.3	1.4
356	100.1	2.4
510	99.4	1.1

[a] Calibration curve was 7.5–75 µg/L carnitine and 30–300 µg/L choline.

(**d**) For this analysis (for either choline or carnitine), given the mass of IS added to the samples and the concentration of IS in the standards, the DF can be shown to be:

$$DF = 3.125/\text{sample weight (g)} \quad (2)$$

(**e**) Thus, the final result (C_x) can be calculated by combining Equations 1 and 2.

Validation Protocol

An SLV protocol was carried out to ensure that the method meets the requirements of the SMPRs for choline (SMPR **2012.013**) and carnitine (SMPR **2012.010**). The SMPRs are summarized in Table 1. The full suite of 11 "SPIFAN matrixes" were used in this SLV. These materials (Table 2**)** were made by the manufacturers specifically for the SPIFAN project to be representative of nearly the entire body of infant and adult nutritional products. By SPIFAN convention, the powders were all prepared as reconstitutions (11.1% by weight), and the indicated aliquot sizes were taken. The three liquid/ready-to-feed (RTF) samples were weighed as-is. The SLV probed the usual parameters of linearity, LOQ/LOD, repeatability, intermediate precision, spike recovery (for accuracy), and agreement of results to independent methods or SRMs.

(**a**) *Linearity*.—On each of 3 different days, calibration curves were prepared for choline and carnitine, and then five independently made standard solutions of differing concentrations were injected as samples. The standard solutions covered the range of the calibration curve from 30 to 300 µg/L choline and from 8 to 80 µg/L carnitine, except the carnitine linearity test included a solution at ½ WS1 and the choline linearity test included a solution that was well above WS3 in concentration. The 3-day mean concentration results from these standard solutions were compared to their nominal concentrations, with an expected agreement of better than ±5%.

(**b**) *LOD/LOQ*.—The adult powder was selected as a suitable placebo in lieu of other possible placebos included in the SPIFAN materials kit. The placebo was weighed at 3.00 g of a 10.93% (w/w) reconstitution and spiked with a known amount of choline (100 µL of a 593.4 µg/mL solution = 59.3 µg, as choline hydroxide in this case) and carnitine (61 µL of a 77.9 µg/mL solution = 4.75 µg). These spike amounts are at the SMPR required LOQ limit: approximately 60 µg/3.0 g RTF = 20 µg/g = 2.0 mg choline hydroxide/100 g RTF; 4.75 µg/3.0 g RTF = 0.16 mg/100 g RTF. The placebo was analyzed seven times to establish a baseline value, and then seven spiked placebos were analyzed and compared to the baseline to determine spike recovery. A spike recovery of 90–110% would establish the spike level as at, or above, the required LOQ.

(**c**) *Accuracy (trueness)*.—SRM 1849a was analyzed with this method, and results were compared to certified values for both choline and carnitine. The 11 SPIFAN matrixes were also spiked with known amounts of choline and carnitine at two different levels, 40 and 80% of the baseline product level, to ensure spike recoveries were in the 90–110% range as required by the SMPR. The spiking was performed on 3 different days, and duplicate spike samples were prepared on each day, at each spiking level. For carnitine, the whole experiment was repeated with acetylcarnitine (Carbosynth LLC, >98% purity, San Diego, CA) instead of L-carnitine, to prove reduction of that species to carnitine with the method's basic hydrolysis. Finally, accuracy of this method was established by comparison of choline results to independent methods. AOAC **999.14** results were obtained from a commercial laboratory (Covance Laboratories, Madison, WI) over 2 days, in duplicate. AOAC **2012.20** results were obtained from the Thermo/Dionex website (5).

(**d**) *Precision*.—Each of the 11 SPIFAN products was analyzed by the method on 6 days in duplicate, for both choline and carnitine, running the method for both free analyte (no microwave digestion or subsequent basic hydrolysis) and total choline/carnitine. The data were collected by two analysts on two different instruments (Waters Acquity TQD, and Xevo TQS), splitting the days evenly among the analyst/instruments. The repeatability precision and the intermediate precision were calculated from the resulting data using analysis of variance calculations from Microsoft (Redmond, WA) Excel.

(**e**) *Specificity*.—Secondary ion transitions were monitored throughout the validation and assessed afterwards for the specificity information they may provide.

Validation Results

Chromatography.—Example chromatograms for the SRM 1849a analysis are shown in Figure 1. Choline and its IS elute first at about 2.5 min followed by the carnitine and its IS at about 4.4 min. Four additional traces are shown in Figure 1 representing the secondary ion transitions that were followed for specificity verification. These data are discussed at the end of this report.

Linearity.—The lowest correlation coefficient observed over the course of the study was 0.9992 for either choline or carnitine. Table 3 shows the results of the 3-day linearity study. The average recovery of independent standard solutions run as samples was 99.1–101.2% at various points along the calibration curve, meeting SMPRs. Note that the method should not be used outside this proven region of linearity. Thus, the lowest concentrations checked (3.4 µg/L carnitine and 30 µg/L choline) become the practical LOQ for the method, even if the

Table 4. LOQ verification in SPIFAN sample 11750017V3 for choline at the SMPR limit of 2 mg/100 g RTF[a]

Sample name	Concn (OH-), mg/kg	Measured spike[b], mg/kg	Theoretical spike, mg/kg	Recovery, %	Spike level, mg/100g RTF
Adult powder-1	60.14				
Adult powder-2	59.76				
Adult powder-3	61.49				
Adult powder-4	60.60				
Adult powder-5	60.87				
Adult powder-6	59.53				
Adult powder-7	59.99				
Unspiked	60.34				
RSD, %	1.1				
Adult powder-LOQ-1	233.5	173.1	178.9	96.8	1.92
Adult powder-LOQ-2	234.9	174.6	178.6	97.8	1.94
Adult powder-LOQ-3	233.3	172.9	176.5	97.9	1.92
Adult powder-LOQ-4	232.5	172.2	179.4	96.0	1.91
Adult powder-LOQ-5	234.1	173.7	180.7	96.1	1.93
Adult powder-LOQ-6	235.5	175.2	179.0	97.9	1.95
Adult powder-LOQ-7	232.2	171.8	179.0	96.0	1.91
Spiked	233.7			96.9	
RSD, %	0.5			0.9	

[a] For this table, the concentrations measured are in terms of choline hydroxide.

[b] The mean unspiked value is subtracted from the individual spiked result.

actual LOQ might be lower due to the sensitivity of the mass spectrometer.

LOQ.—Tables 4 and 5 show the results of spiking a placebo product with amounts of choline and carnitine at the SMPR required LOQ. For choline, a recovery of 96.9% was achieved with excellent precision (0.9% RSD). For carnitine, recovery was 105.9% with a 1.4% RSD. The good recoveries and precision indicate that the method is performing at a level well above the LOQ and the SMPRs are thereby met. Indeed, the inherent choline in this product is below the required 2 mg/100 g (6 mg/100 g powder = 0.66 mg/100 g RTF) and is measured with a 1.1% RSD.

Accuracy (trueness).—SRM 1849a was analyzed by the method on 10 days. The result for free carnitine was 13.1 mg/100 g with an RSD of 2.2%, compared to the certified value of free carnitine of 13.6 mg/100 g, a difference of –3.7%. The method result was within the certified range of 12.2–15.0 mg/100 g. A result of 14.9 mg/100 g (1.6% RSD) was also obtained for total carnitine, but the SRM is not certified for total carnitine.

The result for total choline in SRM 1849a was 102.7 mg/100 g as choline ion, compared to the certified value of 109.0, a difference of –5.8% but within the certified range of 98–120 mg/100 g. NIST obtained 103.2 ± 0.7 mg/100 g for total choline and 14.9 ± 0.1 mg/100 g for free carnitine using its LC/MS method (4). These latter values are closer to the results of the present method, although it is unclear exactly how the microwave digestion step included in the NIST method affects free carnitine results (our free carnitine result was determined after simple water dilution, and our total carnitine result agrees exactly with the individual NIST result in reference 4).

Table 6 shows that there is generally good agreement across the various methods for determination of choline with the exception of the widely used AOAC **999.14**. All four methods compared are quite independent in terms of methodology: microwave-digestion/LC/MS/MS, ion chromatography, enzyme assisted breakdown with colorimetric detection, and HPLC-electrochemical detection. Although this is a limited data set, the obvious recommendation is to avoid using AOAC **999.14** for the analysis of choline in infant formula or adult nutritional products. The other AOAC First Action methods in the table are more accurate over the full breadth of product matrixes presently on the market.

Another LC/MS method (AOAC **2012.17**; 1) was used to confirm the accuracy of AOAC **2014.04** total carnitine results. Twelve internal products (not the SPIFAN set) were analyzed on 8 days by AOAC **2014.04** and on 3–6 days by the reference method. The differences in the mean results for each product ranged from –3.7% to +4.6% with an average of –0.3% (data not shown), indicating the accuracy of this present method for total carnitine.

Tables 7 and 8 show spike recovery data from each of the SPIFAN product matrixes at two different levels for choline and carnitine, including a separate set of experiments to prove the recovery of a primary inherent species, acetylcarnitine. No systematic recovery issues were seen, with overall recoveries close to 100% across all matrixes for choline and carnitine. Acetylcarnitine recoveries appeared to be consistently lower, but still within the SMPR-recommended 90–110%.

Table 5. LOQ verification in SPIFAN sample 11750017V3 for carnitine at the SMPR limit of 0.16 mg/100 g RTF

Sample name	Concn (OH-), mg/kg	Measured spike[a], mg/kg	Theoretical spike, mg/kg	Recovery, %	Spike level, mg/100 g RTF
Adult powder-1	0				
Adult powder-2	0				
Adult powder-3	0				
Adult powder-4	1.1				
Adult powder-5	0				
Adult powder-6	1.3				
Adult powder-7	0				
Unspiked	0				
RSD, %	NA[b]				
Adult powder-LOQ-1	15.22	15.22	14.33	106.2	0.169
Adult powder-LOQ-2	14.95	14.95	14.31	104.5	0.166
Adult powder-LOQ-3	14.90	14.90	14.14	105.4	0.166
Adult powder-LOQ-4	15.30	15.30	14.37	106.4	0.170
Adult powder-LOQ-5	15.00	15.00	14.48	103.6	0.167
Adult powder-LOQ-6	15.48	15.48	14.34	108.0	0.172
Adult powder-LOQ-7	15.36	15.36	14.34	107.1	0.171
Spiked	15.17			105.9	
RSD, %	1.5			1.4	

[a] The mean unspiked value is subtracted from the individual spiked result.

[b] NA = Not applicable.

Table 6. Total Choline (as ion, mg/100g as RTF) method comparison

	6 days × 2				2 days × 2	3 days × 2
Product name	**2014.04**	RSD, %	**2012.20** IC[a]	IC RSD, %	AOAC **999.14**	Abbott IMER[b]
SRM1849a (certified 12.1)[c]	11.4	1.2	10.2	1.8	11.3	10.7
Infant powder milk	16.9	1.8	15.8	2.3	14.7	16.2
Infant powder soy	19.2	2.0	17.8	2.5	18.0	19.3
Infant powder milk part hydrolyzed	17.9	1.8	17.1	2.0	14.9	17.6
Infant powder soy part hydrolyzed	17.0	1.7	16.4	2.1	13.4	17.1
Adult powder low fat	17.4	2.3	16.6	2.1	16.6	16.4
Adult powder milk	4.03	3.2	3.00	3.0	3.00	3.90
Child formula powder	5.45	2.1	4.96	2.7	4.79	5.20
Infant elemental powder	8.16	1.9	7.70	2.1	3.78	7.95
Infant RTF milk	20.9	2.1	20.1	2.5	18.0	21.0
Adult RTF high protein	47.2	1.5	46.6	2.4	44.8	46.1
Adult RTF high fat	51.5	1.3	51.1	2.5	50.9	51.9

[a] Thermo Fisher Scientific ion chromatography **2012.20** SLV, 6 days in duplicate, from published Application Note (5).

[b] Abbott Nutrition unpublished method employing digestion at 90°C for 3 h, immobilized enzyme reactor (IMER) column using choline oxidase to convert choline to betaine and peroxide, followed by electrochemical detection of peroxide.

[c] The certified value for SRM 1849a is 1090 mg/kg as powder, or 12.1 mg/100 g RTF as total choline ion.

Table 7. Spike recovery of choline (ion) in SPIFAN matrixes at two different levels

		Spike recovery			
		+40% Native		+80% Native	
Product	Native level mg/100 g as RTF	Avg., %	RSD, %	Avg., %	RSD, %
Infant powder milk	16.6	101.3	2.0	97.8	1.7
Infant powder soy	19.1	95.8	5.2	92.8	6.7
Infant powder milk part hydrolyzed	17.6	95.7	5.8	96.8	3.5
Infant powder soy part hydrolyzed	17.1	94.0	6.2	98.8	4.4
Adult powder low fat	17.3	97.0	6.4	98.9	3.2
Adult powder milk	<LOQ[a]	<LOQ	NA[b]	90.5	6.9
Child formula powder	5.44	104.1	0.7	100.3	6.0
Infant elemental powder	8.42	99.2	3.0	100.1	2.3
Infant RTF milk	21.42	102.2	4.7	99.3	4.0
Adult RTF high protein	48.35	102.4	7.7	101.1	2.4
Adult RTF high fat	53.20	104.2	4.3	97.4	2.8
Mean		99.6		97.6	

[a] Further spiking results with adult milk powder are given in Table **2014.04K** and **L**.

[b] NA = Not applicable.

Table 8. Spike recovery of carnitine and acetylcarnitine in SPIFAN matrixes at two different levels

		Spike recovery			
		+40% Native		+80% Native	
Product	Native level mg/100 g as RTF	Avg., %	RSD, %	Avg., %	RSD, %
	Carnitine				
Infant powder milk	1.71	98.3	2.2	96.2	2.4
Infant powder soy	0.898	97.4	1.0	98.6	1.9
Infant powder milk part hydrolyzed	1.12	99.6	4.1	98.0	4.7
Infant powder soy part hydrolyzed	1.01	96.8	1.6	98.9	1.7
Adult powder low fat	0.00	102.3	3.4	99.4	1.1
Adult powder milk	0.00	100.6	0.6	98.8	0.6
Child formula powder	5.39	100.2	2.1	97.5	4.2
Infant elemental powder	1.51	100.2	3.4	95.5	3.2
Infant RTF milk	2.68	99.7	4.0	95.8	4.0
Adult RTF high protein	15.5	97.3	1.6	98.7	1.7
Adult RTF high fat	21.6	97.5	4.8	98.0	2.0
Mean		99.1		97.8	
	Acetycarnitine (determined separately)				
Infant powder milk	1.69	103.0	2.5	90.7	4.4
Infant powder soy	0.90	96.7	4.6	95.3	3.0
Infant powder milk part hydrolyzed	1.10	94.9	8.0	91.1	5.0
Infant powder soy part hydrolyzed	1.00	94.6	3.9	91.1	4.2
Adult powder low fat	0.00	98.6	4.5	96.0	5.2
Adult powder milk	0.00	96.0	4.1	97.0	2.0
Child formula powder	5.42	97.8	2.8	98.3	3.2
Infant elemental powder	1.53	100.1	6.1	95.9	3.9
Infant RTF milk	2.65	101.7	2.3	98.7	1.9
Adult RTF high protein	15.5	92.8	5.1	98.4	2.3
Adult RTF high fat	21.5	98.4	6.7	95.9	1.0
Mean		97.7		95.3	

Table 9. Repeatability and intermediate precision for total carnitine and total choline (6 days in duplicate)

Product	Concn level mg/100 g RTF	RSD_r, %	RSD_{iR}, %
Total choline			
Infant powder milk	16.9	1.2	1.7
Infant powder soy	19.2	0.9	1.7
Infant powder milk part hydrolyzed	17.9	1.0	1.8
Infant powder soy part hydrolyzed	17.0	0.9	1.8
Adult powder low fat	17.4	1.4	1.9
Adult powder milk	4.03	2.3	3.0
Child formula powder	5.45	1.3	2.5
Infant elemental powder	8.16	1.9	2.0
Infant RTF milk	20.9	0.8	2.1
Adult RTF high protein	47.2	1.4	1.4
Adult RTF high fat	51.5	1.0	1.4
Overall mean, %		1.3	1.9
Total carnitine			
Infant powder milk	1.71	1.5	1.5
Infant powder soy	0.899	0.8	2.2
Infant powder milk part hydrolyzed	1.14	0.9	1.8
Infant powder soy part hydrolyzed	1.01	1.3	2.1
Adult powder low fat	0.039	<LOQ	< LOQ
Adult powder milk	0.0076	< LOQ	< LOQ
Child formula powder	5.44	0.7	2.0
Infant elemental powder	1.54	1.5	1.5
Infant RTF milk	2.69	1.6	1.6
Adult RTF high protein	15.6	1.0	1.4
Adult RTF high fat	21.5	1.7	1.7
Overall mean, %		1.2	1.8

Table 10. Repeatability and intermediate precision for free carnitine and free choline (6 days in duplicate)

Product	Concn level mg/100g RTF	RSD_r, %	RSD_{iR}, %
Free choline			
Infant powder milk	12.8	0.7	2.1
Infant powder soy	13.9	1.3	2.7
Infant powder milk part hydrolyzed	13.4	2.0	2.3
Infant powder soy part hydrolyzed	14.2	1.1	2.1
Adult powder low fat	15.1	1.5	2.4
Adult powder milk	0.36	1.7	19
Child formula powder	4.39	1.4	5.4
Infant elemental powder	7.59	0.9	1.4
Infant RTF milk	12.1	0.7	0.9
Adult RTF high protein	42.3	1.3	1.3
Adult RTF high fat	46.8	1.2	1.4
Overall mean, %		1.3	2.2
Free carnitine			
Infant powder milk	1.50	1.4	3.4
Infant powder soy	0.890	1.5	3.8
Infant powder milk part hydrolyzed	0.882	1.3	1.7
Infant powder soy part hydrolyzed	1.00	1.3	2.7
Adult powder low fat	0.0200	<LOQ	<LOQ
Adult powder milk	0.0100	<LOQ	<LOQ
Child formula powder	5.48	1.8	2.7
Infant elemental powder	1.54	0.9	1.4
Infant RTF milk	2.63	2.0%	2.0%
Adult RTF high protein	15.7	1.5	1.5
Adult RTF high fat	21.9	1.6	1.6
Overalll mean, %		1.5	2.3

Precision.—Within-day precision across the SPIFAN matrix set generally fell in the 0–2% RSD range with the intermediate precision across 6 days being about 1% higher. These data are summarized in Table 9 (for total choline/carnitine) and 10 (for free choline/carnitine) and easily meet the SMPRs for repeatability (*see* Table 1). Reproducibility was not tested with this SLV, but the excellent intermediate precision across two analysts and two instruments in this study indicate that the requirement of <15% RSD_R would likely be met in a multilaboratory study.

Free versus total.—Table 11 summarizes the results from the SPIFAN matrixes in terms of the free versus total amounts. In all cases, the result for total choline was substantially greater than that for free choline. Discounting the results for carnitine in the Adult Powders, which had very low levels, it could be said that for most products, the total carnitine level equals the free carnitine level. The exception is the SRM 1849a material and some of the products containing milk protein. The significance of this is that, in most cases, manufacturing laboratories are interested in free carnitine (how much is added) and total choline results for their products. A single analysis of the final digested solution will suffice to measure both of these parameters.

Specificity.—The choline-d_9 and carnitine-d_3 labeled isotopes were checked for the presence of any response at the primary transition of the native compound, and only a negligible signal was observed (data not shown). Conversely, during an internal validation with 12 Abbott products (not the SPIFAN set), the internal standards were not added to the samples on 1 day and negligible response was observed at the primary transitions of the internal standards, indicating these transitions are specific (data not shown).

Throughout the course of an internal validation of this method in the authors' laboratory, data were collected at confirmatory transitions for both choline and carnitine. These transitions are included in Figure 1 along with primary transitions used for quantitation of SRM 1849a. For carnitine, the confirmatory transition used was 162→85 amu for the native compound and 165→85 amu for the IS. For choline, it was 104.2→45 amu for the native compound and 113.4 → 45 amu for the IS. The data were examined after the validation to see how the ratios of response at the confirmatory transition relative to response

Table 11. Free versus total choline/carnitine in the SPIFAN matrix set

	Choline (as OH, mg/kg)					Carnitine mg/kg				
	Dilute-injection (free)		MW digestion (total)			Dilute-injection (free)		MW digestion (total)		
Product name	*n* = 6 × 2 × 2	Avg. RSD, %	*n* = 6 × 2 × 2	Avg. RSD, %	Free/ total, %	*n* = 6 × 2 × 2	Avg. RSD, %	*n* = 6 × 2 × 2	Avg. RSD, %	Free/ total, %
SRM1849a (*n* = 5)	902	1.6	1186	1.2	76	130	2.2	149	1.6	88
Infant powder milk	1345	2.1	1768	1.8	76	136	3.2	154	1.4	88
Infant powder soy	1463	2.5	2000	2.0	73	80.3	3.3	80.7	1.7	100
Infant powder milk part hydrolyzed	1403	2.0	1873	1.8	75	80.2	2.2	103	1.7	78
Infant powder soy part hydrolyzed	1490	2.1	1789	1.7	83	91.0	2.6	90.7	1.6	100
Adult powder low fat	1587	2.1	1819	2.3	87	1.6	84	3.4	46	48
Adult powder milk	37.2	17.0	423	3.2	9	0.5	140	0.7	56	66
Child formula powder	459	4.5	572	2.1	80	493	2.6	490	1.8	101
Infant elemental powder	798	1.6	857	1.9	93	138	1.4	138	1.9	100
Infant RTF milk	141	1.5	243	2.1	58	26.2	2.0	26.8	1.6	98
Adult RTF high protein	494	1.1	550	1.5	90	157	1.4	155	1.4	102
Adult RTF high fat	546	1.6	601	1.3	91	220	2.0	215	1.3	102

Table 12. Summary of daily carnitine and choline ion ratio (response at primary transition/response at confirmatory transition): data averaged across analysts, free/total results, and sample types[a]

Carnitine									
TQD					TQS				
Day	N	Mean	SD	RSD, %	Day	N	Mean	SD	RSD, %
1	37	2.061	0.042	2.02	1	19	0.609	0.009	1.52
2	37	1.999	0.037	1.87	2	19	0.608	0.012	2.02
3	37	1.858	0.030	1.64	3	19	0.587	0.006	1.10
4	37	1.654	0.040	2.44	4	19	0.588	0.007	1.17
5	38	1.862	0.050	2.66	5	21	0.617	0.019	3.06
6	37	1.874	0.035	1.88	6	21	0.631	0.019	3.00
7	37	1.842	0.031	1.69	7	18	0.648	0.008	1.24
8	37	1.820	0.024	1.34	8	24	0.670	0.024	3.55
					9	37	0.624	0.006	0.91
					10	37	0.627	0.005	0.78
					11	38	0.632	0.010	1.55
					12	37	0.638	0.004	0.61
Pooled				1.98					1.83
Choline									
1	38	1.436	0.010	0.73	1	20	1.532	0.021	1.35
2	38	1.448	0.020	1.41	2	20	1.598	0.022	1.38
3	38	1.446	0.021	1.42	3	20	1.620	0.027	1.64
4	38	1.322	0.019	1.47	4	20	1.583	0.017	1.10
5	38	1.423	0.013	0.92	5	21	3.098	0.096	3.10
6	38	1.436	0.014	0.97	6	21	3.218	0.088	2.74
7	38	1.414	0.016	1.10	7	18	3.136	0.044	1.41
8	38	1.397	0.013	0.94	8	24	3.535	0.192	5.43
					9	38	1.199	0.015	1.27
					10	38	1.194	0.023	1.93
					11	38	1.218	0.018	1.50
					12	38	1.197	0.018	1.50
Pooled				1.15					2.27

[a] Obtained during authors' internal laboratory validation using other products, not the SPIFAN kit.

at the primary transition varied on a given day and a given instrument. Table 12 shows the results.

Daily ratios for carnitine were, overall, more consistent than those for choline, especially for data collected on the TQS instrument. For 4 of the 12 days of TQS choline data, the transition ratio was approximately twice that observed on the remaining days. There is no obvious explanation for this shift. Within-day uniformity was generally good on those days (within an acceptable range), although on 3 of those 4 days the ratio RSDs were modestly larger. Despite the large shift in the ion ratio on those days, however, there was no other indication that data quality was compromised. Overall, within-day RSD ranges were carnitine/TQD: 1.3–2.7%, carnitine/TQS: 0.6–3.6%, choline/TQD: 0.7–1.5%, and choline/TQS: 1.1–5.4%. These are consistent with expectations, generally, for LC/MS/MS methods.

Because of the differences between instrument platforms as well as day-to-day shifts, it is not possible to assign meaningful ranges appropriate for all instruments on all days. A better alternative is to base the expected ratio in the samples to that determined for the current daily calibration standards, and to use some multiple of the SD of the ratio determined for the three standards to set a limit for the ratio expected in the samples. Thus, if a particular sample's ratio was 2–3 SD away (the exact setting is arbitrary) from the typical ratio in the daily calibration standards, that would be flagged as a possibly invalid result (caused by a coeluting molecule that happened to have exactly the same parent and primary daughter ion mass) and investigated (e.g., diluted and reanalyzed). The AOAC ERP has previously suggested this type of approach for MS/MS platforms to deliver the most highly specific methods to the analytical community. As reasonable as this approach sounds, we have found it very difficult to put into practice for LC/MS/MS methods. The flags raised can disrupt laboratory operations, and the subsequent investigation/retest is often ambiguous as to whether the result is more accurate, or even statistically different from the flagged result. Given the excellent performance of AOAC **2014.04** and good agreement among different methods on a variety of matrixes, we conclude that confirmatory ion transitions are not necessary for this method.

Conclusions

An SLV of AOAC **2014.04** has been performed, and the method has met all the requirements for precision, accuracy, linearity, LOQ, and specificity as outlined in SMPR **2012.010** for carnitine. The method simultaneously determines choline, and the data show that it has also met the requirements of SMPR **2012.013** for that analyte.

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Vitamin B_{12} in Infant, Adult, and Pediatric Formulas by HPLC-UV and Column Switching: Collaborative Study, Final Action 2011.10

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AOAC First Action Method 2011.10, Vitamin B_{12} in Infant and Pediatric Formulas and Adult Nutritionals, was collaboratively studied. This method uses a pH 4.5 sodium acetate buffer and potassium cyanide at 105°C to extract and convert all biologically active forms of vitamin B_{12} present to cyanocobalamin; octylsilyl (C_8) or C_{18} SPE cartridges to purify and concentrate cyanocobalamin; a combination of size-exclusion and RPLC to isolate cyanocobalamin; and visible absorbance at 550 nm to detect and quantitate cyanocobalamin in infant, pediatric, and adult nutritionals with vitamin B_{12} concentrations greater than 0.025 µg/100 g ready-to-feed (RTF) liquid. During this collaborative study, nine to 11 laboratories from eight different countries analyzed blind duplicates of 12 infant, pediatric, and adult nutritional formulas. Per the AOAC Expert Review Panel (ERP) on Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Nutrient Methods the method demonstrated acceptable repeatability and reproducibility and met SPIFAN *Standard Method Performance Requirements* (SMPRs®) for the majority of product matrixes analyzed. Vitamin B_{12} SPIFAN SMPRs for repeatability were ≤15% RSD at vitamin B_{12} concentrations of 0.01 µg/100 g RTF liquid and ≤7% RSD at vitamin B_{12} concentrations of 0.2–5.0 µg/100 g RTF liquid. Vitamin B_{12} SPIFAN SMPRs for reproducibility were ≤11% RSD in products with vitamin B_{12} concentrations ranging from 0.3 to 5.0 µg/100 g RTF liquid. During this collaborative study, the RSD_r ranged from 2.98 to 9.77%, and the RSD_R ranged from 3.54 to 19.5%. During previous single-laboratory validation studies, the method LOQ was estimated to be 0.025 µg/100 g RTF liquid.

Vitamin B_{12} is a generic term used to describe all cobalamins that demonstrate antipernicious anemia activity. These compounds are corrinoids, tetrapyrrole structures where the cobalt ion is chelated by four pyrrole nitrogens. The fifth coordinate covalent bond to cobalt is with a nitrogen of the dimethylbenzimidazole moiety, while the sixth position may be occupied by a cyanide, 5'-deoxyadenosyl, methyl, water, hydroxyl, nitrite, ammonia, or sulfite ligand (1).

The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) has defined vitamin B_{12} as any cobalt-containing corrinoid with the biological activity of cyanocobalamin, including cyanocobalamin, aquocobalamin, hydroxycobalamin, methylcobalamin, and adenosylcobalamin. Most nutritional products are fortified with cyanocobalamin, a synthetic form of vitamin B_{12}, because of its stability and commercial availability. Methylcobalamin and 5'-deoxyadenosylcobalamin occur naturally and are known to exhibit metabolic activity in humans. Naturally occurring vitamin B_{12} originates solely from synthesis by bacteria and other microorganisms growing in soil, water, or sewage and in the rumen and intestinal tract of animals (2).

A new chromatographic method capable of determining vitamin B_{12} in infant, adult, and pediatric formula powders, ready-to-feed (RTF) liquids, and liquid concentrates was needed after SPIFAN determined that traditional microbiological vitamin B_{12} methods were not precise and accurate enough to meet the nutrient specification requirements of infant, pediatric, and adult nutritionals. The SPIFAN vitamin B_{12} working group developed *Standard Method Performance Requirements* (SMPRs®) that were then approved by SPIFAN (3).

In June 2011, the AOAC Expert Review Panel (ERP) on SPIFAN nutrient methods granted First Action status to multiple vitamin B_{12} methods, AOAC **2011.08** (4), **2011.09** (5), AOAC **2011.10** (6), and **2016.16** (7). Single-laboratory validations (SLVs) were completed for AOAC **2011.08** and AOAC **2011.10** (8), and in March 2013, AOAC **2011.10** was selected by the ERP for further evaluation in a multilaboratory collaborative study to determine method reproducibility.

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The method was approved by the AOAC *Official Methods Board* as Final Action. *See* "Standards News," (2014) *Inside Laboratory Management*, November/December 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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AOAC **2011.10** uses a pH 4.5 sodium acetate buffer and potassium cyanide at 105°C to extract and convert all forms of vitamin B_{12} present to cyanocobalamin, C_8 or C_{18} SPE cartridges to purify and concentrate cyanocobalamin, a combination of size-exclusion and RPLC to isolate cyanocobalamin, and visible absorbance at 550 nm to detect and quantitate cyanocobalamin. This method is applicable to the determination of vitamin B_{12}, including cyanocobalamin, aquocobalamin, hydroxycobalamin, methylcobalamin, and adenosylcobalamin, in all forms of infant, adult, and pediatric formula (powders, RTF liquids, and liquid concentrates.)

Multilaboratory Collaborative Study

Initially 17 laboratories expressed interest in participating in the AOAC **2011.10** vitamin B_{12} collaborative study, but only 11 laboratories were able to complete the study. The 11 participating laboratories were located in eight different countries. The remaining six laboratories were not able to participate because of time and resource constraints and issues with the importation of samples into their countries. Two of the participating laboratories only received partial sample shipments.

Before actual multilaboratory collaborative study samples were analyzed, each participating laboratory was asked to analyze two practice samples in duplicate to identify and resolve any testing issues that the laboratories may have had executing the method. The practice samples included National Institute of Standards and Technology (NIST; Gaithersburg, MD) standard reference material (SRM) 1849a and a high protein adult nutritional RTF product. After approval of the practice sample results by the study directors, laboratories began testing the study samples.

Blind duplicates of the 12 SPIFAN matrixes were shipped to each participating laboratory. The matrixes included SRM 1849a, an adult nutritional milk protein-based powder, an infant formula partially hydrolyzed milk-based powder, an infant formula partially hydrolyzed soy-based powder, an adult nutritional low-fat powder, a child formula powder, an infant elemental powder, an infant formula milk-based powder, an infant formula soy-based powder, an infant formula milk-based RTF liquid, an adult high-fat nutritional RTF liquid, and an adult high protein nutritional RTF liquid.

Participants were asked to reconstitute all powders prior to analysis. SRM 1849a 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. Participants were asked to split the 24 test samples into two groups of 12 according to the data reporting sheets included in the protocol and to test each group on a separate day. Although the original AOAC **2011.10** First Action method allowed for the use of different size SPE cartridges, depending on the sample type being analyzed, collaborative study participants were required to test all samples with cartridges containing at least 900 mg resin since the study samples were blinded. Most laboratories used the 900 mg octylsilyl (C_8) or C_{18} cartridges referenced in the method, but one participating laboratory used 1 g C_{18} cartridges.

Upon completion of the sample analyses, participating laboratories were asked to send all of their data to the study directors. This included all standard and sample chromatograms for the instrument check, practice sample analyses, test sample analyses, standard curve information, calculations, and completed reporting of analysis forms with dilution and sample weights. Participants were also asked to report any deviations to the method and any relevant comments based on their experiences with the method.

All data were statistically analyzed using AOAC INTERNATIONAL guidelines to determine overall mean, repeatability SD (s_r), RSD_r, reproducibility SD (s_R), RSD_R, and Horwitz ratio (HorRat; 9). Cochran ($P = 0.025$, one-tail) and Grubbs (single and double, $P = 0.025$, two-tail) tests were used to determine statistical outliers.

Vitamin B_{12} SPIFAN SMPRs for repeatability were ≤15% RSD at vitamin B_{12} concentrations of 0.01 µg/100 g RTF liquid and ≤7% RSD at vitamin B_{12} concentrations of 0.2–5.0 µg/100 g RTF liquid. Requirements for reproducibility were ≤11% RSD in products with vitamin B_{12} concentrations ranging from 0.3 to 5.0 µg/100 g RTF liquid.

Method

A few minor modifications were made to AOAC Official First Action method **2011.10** before it was sent to the study participants in the collaborative study protocol. These changes included increasing the concentration of the sodium acetate buffer from 0.1 to 0.25 M, adding more RP column options, providing guidance for the preparation of samples containing free amino acids or no intact protein, and providing guidance for choosing appropriate size SPE cartridges.

After completion of the study, the modifications noted above were incorporated in the Final Action method along with a few additional modifications based on study results and feedback from study participants and the ERP. In addition to the modifications listed above, procedures for safely handling potassium cyanide, qualifying SPE cartridges, and establishing appropriate elution gradients to adequately resolve vitamin B_{12} on the RP column were added to the Final Action method. The option for using SPE cartridges smaller than 900 mg and a guidance for choosing appropriate size SPE cartridges were removed from the method since many laboratories may not have enough information about the samples that they are testing to use smaller size SPE cartridges.

AOAC Official Method 2011.10
Vitamin B_{12} in Infant and Pediatric Formulas and Adult Nutritionals
HPLC
First Action 2011
Final Action 2014

ISO-AOAC Method

(Applicable to the determination of vitamin B_{12} in infant and pediatric formulas and adult nutritionals.)

Caution: Refer to Material Safety Data Sheets of chemicals prior to use. Use the suggested personal protective equipment and follow good laboratory practices.

Note: Potassium cyanide is highly toxic. When handling this chemical, wear gloves and appropriate personal protective equipment. Weigh chemical and dispense solutions in a fume hood. Perform test in a well-ventilated area. Treat sample waste with sodium hypochlorite and dispose of waste according to

Table 2011.10A. SLV repeatability precision data for vitamin B_{12}

Sample type	No. of replicates (duplicates on multiple days)	Mean, µg/100 g RTF	SD_r	RSD_r, %
Infant formula (NIST SRM 1849a)	18	42.4[a]	1.39	3.27
Infant formula powder soy-based	12	0.415	0.0150	3.62
Infant formula powder milk-based	12	0.236	0.0083	3.52
Infant formula RTF milk-based	12	0.355	0.0083	2.34
Infant formula powder partial hydrolyzed milk-based	12	0.377	0.0132	3.50
Infant formula powder partial hydrolyzed soy-based	12	0.257	0.0090	3.51
Adult nutritional powder milk-based	12	0.299	0.0141	4.72
Adult nutritional RTF high protein	12	1.13	0.0250	2.21
Child formula powder	12	0.955	0.0225	2.36
Adult nutritional RFT high fat	12	1.65	0.0463	2.81
Infant elemental powder	12	0.540	0.0223	4.13
Adult nutritional powder low fat	12	0.666	0.0187	2.81

[a] Results in µg/kg powder.

Table 2011.10B. SLV accuracy data for vitamin B_{12}—spike recoveries at 100% of native level

Sample type	No. of replicates (duplicates on multiple days)	Native level, µg/100 g RTF	Recovery, %	RSD, %
Infant formula (NIST SRM 1849a)	4	42.6[a]	95.7	5.32
Infant formula powder soy-based	6	0.404	98.2	5.32
Infant formula powder milk-based	4	0.236	104	4.32
Infant formula RTF milk-based	6	0.326	96.4	8.17
Infant formula powder partial hydrolyzed milk-based	4	0.347	98.2	6.34
Infant formula powder partial hydrolyzed soy-based	6	0.248	95.7	2.51
Adult nutritional powder milk-based	4	0.298	97.4	3.86
Adult nutritional RTF high protein	6	1.12	98.6	6.76
Child formula powder	4	0.109	98.2	7.98
Adult nutritional RFT high fat	4	1.59	102	1.61
Infant elemental powder	6	ND[b]	105	4.45
Adult nutritional powder low fat	4	0.639	95.1	4.38

[a] Results in µg/kg powder.

[b] ND = Not detected.

Table 2011.10C. Interlaboratory study results for vitamin B_{12}

Sample type	Total No. labs	Total No. replicates	Mean, µg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat[a]
Infant formula (NIST SRM 1849a)	10	20	43.7[b]	3.01	3.86	6.90	8.84	0.34
Infant formula powder soy-based	10	20	0.428	0.0208	0.0305	4.85	7.13	0.20
Infant formula powder milk-based	9	18	0.227	0.0111	0.0202	4.90	8.90	0.22
Infant formula RTF milk-based	9	18	0.272	0.0257	0.0427	9.46	15.7	0.40
Infant formula powder partial hydrolyzed milk-based	11	22	0.373	0.0200	0.0694	5.35	18.6	0.50
Infant formula powder partial hydrolyzed soy-based	10	20	0.250	0.0244	0.0487	9.77	19.5	0.50
Adult nutritional powder milk-based	10	20	0.300	0.0270	0.0416	8.99	13.8	0.36
Adult nutritional RTF high protein	10	20	1.08	0.0730	0.190	6.74	17.5	0.55
Child formula powder	8	16	0.967	0.0289	0.0342	2.98	3.54	0.11
Adult nutritional RTF high fat	9	14	1.48	0.122	0.171	8.23	11.5	0.38
Infant elemental powder	9	18	0.543	0.0169	0.0603	3.11	11.1	0.32
Adult nutritional powder low fat	10	20	0.636	0.0348	0.0587	5.47	9.23	0.27

[a] HorRat = RSD/PRSD; PRSD = $2C^{-0.15}$ (C = concentration by mass fraction).

[b] Results in µg/kg powder.

local, state, and federal regulations.

See Tables **2011.10A–C** for results of the single-laboratory validation and interlaboratory study supporting acceptance of the method.

A. Principle

Vitamin B_{12} is extracted from samples using sodium acetate buffer (pH 4.5) and potassium cyanide at 105°C. Extracts are purified and concentrated with C_8 or C_{18} SPE cartridges and analyzed with size-exclusion and RP chromatography. Determination of B_{12} is made by LC with UV at 550 nm.

B. Apparatus and Materials

(a) *HPLC system*.—Gradient system with switching valve and additional isocratic pump and a UV-Vis detector equipped with a tungsten lamp (capable of monitoring at 550 nm wavelength). Autosampler capable of injecting 900 µL to 2 mL sample.

(b) *Column*.—Analytical size-exclusion column 4 µm, 250 × 9.4 mm (Zorbax GF-250, P/N 884973-901; www.chem.agilent.com), 5 µm, 300 × 8 mm (Showa Denko America, Inc., New York, NY) Protein KW-802.5, P/N F6989000), or equivalent.

(c) *Column*.—Analytical C_{18} column 3 µm, 100 × 4.6 mm (Thermo Scientific Aquasil P/N 77503-104630; www.thermoscientific.com) with C_{18} drop-in guard cartridges 3 µm, 10 × 4.6 mm (Thermo Scientific Aquasil P/N 77503-014001), Epic phenyl hexyl, 3 µm, 120 Å, 100 × 4.6 mm (ES Industries P/N 125191-EPHX; www.esind.com) with appropriate guard cartridge, or equivalent.

(d) *Oven*.—Capable of maintaining temperatures of 95 ± 5°C and 105 ± 5°C.

(e) *pH meter*.—With calibration buffer.

(f) *Analytical balance*.—Capable of weighing to 0.00001 g.

(g) *Beakers*.—Glass, assorted sizes.

(h) *Bottle top dispenser*.—Capable of dispensing 30 mL.

(i) *Cylinders*.—Graduated glass, assorted sizes.

(j) *Desiccator*.

(k) *Erlenmeyer flasks*.—125 mL or equivalent glassware.

(l) *Filter paper*.—Whatman 2V (www.whatman.com) or equivalent.

(m) *Funnels*.—Plastic, suitable to use with filter paper.

(n) *Gloves*.

(o) *Pipettor*.—Variable volume, 100–1000 µL.

(p) *Shields*.—Yellow or clear shields with a cutoff of at least 385 nm.

(q) *SPE cartridges*.—C_8 900 mg (Alltech/Grace Davison, Bannockburn, IL; P/N 20966), C_{18} 900 mg (Alltech/Grace Davison; P/N 20942), or equivalent. *See* **E** for SPE cartridge qualification.

(r) *Syringes*.—Disposable, assorted sizes.

(s) *Syringe filters*.—0.45 µm nylon.

(t) *Vacuum manifold*.—24 ports with stopcocks or equivalent.

(u) *Volumetric pipets*.—Assorted sizes.

(v) *Volumetric flasks*.—Assorted sizes.

C. Reagents

(a) *Acetic acid*.—Glacial, ACS.

(b) *Acetonitrile*.—HPLC grade.

(c) *Drierite*.—Desiccant, anhydrous calcium sulfate, 8 mesh.

(d) *Ethanol*.—Reagent alcohol, 95%, denatured.

(e) *Formic acid*.—88%, ACS.

(f) *Laboratory water*.—≥15 MΩ·cm.

(g) *Potassium cyanide*.—≥97%, ACS.

(h) *Riboflavin*.—≥96%, ACS.

(i) *Sodium acetate anhydrous or sodium acetate trihydrate*.—ACS.

(j) *Taka-diastase*.—Accurate Chemical Co. (www.accuratechemical.com) or equivalent.

(k) *Triethylamine (TEA)*.—HPLC grade.

(l) *Vitamin B_{12} (cyanocobalamin) standard*.—USP reference, official lot number (refer to USP catalog for current lot). Store in desiccator protected from white light. (*Note*: *See* standard label for purity).

D. Solution and Standard Preparation

All solutions can be scaled up or down for convenience provided good laboratory practices are observed. Solutions can be stored at 2–30°C in tight, inert containers unless otherwise noted.

(a) *Solutions*.—(*1*) *Mobile phase A*.—4.0 mL TEA diluted with 1000 mL water and pH adjusted to 5–7 with about 1.25 mL concentrated formic acid. Expiration 1 week.

(*2*) *Mobile phase B*.—4.0 mL TEA and 250 mL acetonitrile diluted with 750 mL laboratory water and pH adjusted to 5–7 with about 1.25 mL concentrated formic acid. Expiration 1 week in tightly stoppered container.

(*3*) *Mobile phase C*.—4.0 mL TEA and 750 mL acetonitrile diluted with 250 mL laboratory water and pH adjusted to 5–7 with about 1.25 mL concentrated formic acid. Expiration 1 week in tightly stoppered container.

(*4*) *Mobile phase D (2.5% acetonitrile in water)*.—50 mL acetonitrile diluted to 2000 mL with laboratory water. Expiration 1 week in tightly stoppered container.

(*5*) *10% Acetonitrile in water*.—150 mL acetonitrile diluted to 1500 mL with laboratory water. Expiration 1 month in tightly stoppered container.

(*6*) *30% Acetonitrile in H_2O SPE elution solvent*.—30.0 mL acetonitrile diluted to 100 mL with laboratory water. Expiration 1 month in tightly stoppered container.

(*7*) *50% Acetonitrile in water, column cleaning and storage solution*.—500 mL acetonitrile diluted to 1000 mL in a volumetric flask. Expiration 6 months.

(*8*) *25% Ethanol*.—50 mL ethanol diluted to 200 mL with laboratory water. Expiration 1 year in tightly stoppered container.

(*9*) *0.40% Potassium cyanide for samples with 5 mL final dilution volume*.—Dissolve 0.02 g potassium cyanide in and dilute to 5 mL with 0.25 M sodium acetate buffer. Make fresh daily before use.

(*10*) *1% Potassium cyanide*.—Dissolve 0.25 g potassium cyanide in and dilute to 25 mL with laboratory water. Prepare fresh daily before use.

(*11*) *Resolution test solution for determining appropriate gradient conditions when a new analytical column is installed*.—Weigh about 0.005 g riboflavin onto a weighing paper. Transfer to a 100 mL volumetric flask and dilute to volume with 10% acetonitrile solution. Stir to dissolve. Mix equal amounts of

riboflavin solution with the highest concentration vitamin B_{12} working standard. Expiration: 1 week.

(*12*) *0.25 M Sodium acetate buffer*.—Dissolve 41 g sodium acetate anhydrous or 68 g sodium acetate trihydrate in approximately 1800 mL laboratory water. Adjust pH to 4.50 with concentrated acetic acid. Dilute to 2000 mL with laboratory water. Expiration 3 months.

(*13*) *6% Taka-diastase*.—Dissolve 0.6 g taka-diastase in 10 mL water. Prepare fresh daily before use.

(b) *Standards*.—Prepare all standards in volumetric glassware under UV shielded fluorescent lights and store at 2–8°C in tightly stoppered volumetric flasks.

(*1*) *Vitamin B_{12} stock standard (10 000 µg/L)*.—Accurately weigh, to 0.00001 g, the appropriate amount of vitamin B_{12} USP reference standard to give a stock standard concentration of 10 000 µg/L. Dissolve in and dilute to 100 mL with 25% ethanol. Expiration 6 months.

Use the following equation to calculate the amount of vitamin B_{12} reference standard that should be weighed:

$$S_w = 10\,000 \times 0.1 \times 1/P$$

where S_w = amount of vitamin B_{12} standard to be weighed in mg; 10 000 = desired stock standard concentration in µg/L; 0.1 = dilution volume in L; P = purity of the USP reference standard in µg cyanocobalamin/mg of the standard. *See* standard label.

(*2*) *Vitamin B_{12} intermediate standard (1000 µg/L)*.—Dilute 10 mL vitamin B_{12} stock standard solution to 100 mL with laboratory water. Expiration 1 week.

(*3*) *Vitamin B_{12} working standards (2.5–25 µg/L)*.—Dilute 0.5, 1, 2, 3, 4, and 5 mL vitamin B_{12} intermediate standard solution to 200 mL with 10% acetonitrile. Expiration 1 month.

E. Procedure

Prepare all samples under UV shielded fluorescent lights. Mix or stir products before sampling to ensure all product samples are uniform and representative. Store prepared product samples up to 14 days after preparation in tightly stoppered volumetric flasks at 2–8°C.

(a) *SPE cartridge qualification*.—To establish SPE cartridge equivalency or to verify the suitability of new lots of cartridges: (*1*) Prepare a solution containing 160 µg/L vitamin B_{12} in water. (*2*) Prepare three samples from one representative product that contains the highest amount of protein of any product that will be analyzed with this method following steps **E(b)**(*1*) and (*2*) of the sample preparation procedure described below. (*3*) Combine all extracted sample filtrates. Accurately transfer 1 mL solution prepared in step (*1*) to 80 or 100 mL of sample filtrate (spiked sample), and accurately transfer 1 mL water to 80 or 100 mL of sample filtrate (unspiked sample).

(*4*) Continue preparing the spiked and unspiked sample using the sample cleanup and concentration, **E(b)**(*3*), and final dilution, **E(b)**(*4*), procedures described in the sample preparation procedure below. (*5*) Analyze the two samples chromatographically. (*6*) Calculate the vitamin B_{12} concentration of the spiked and unspiked samples and calculate the spike recovery. (*7*) In order for the cartridges to be considered acceptable, spike recoveries should be ≥90%.

(b) *Sample preparation for infant, pediatric, and adult nutritional products*.—(*1*) *Sampling*.—Mix all products thoroughly before sampling. Reconstitute nonhomogeneous powders per label instructions. Weigh the appropriate amount of product (±10%) into a 100 mL volumetric flask and record the weight to at least four significant figures. Typical weights are 20 g for adult and pediatric RTF liquids and reconstituted powders, 25 g for infant RTF liquids and reconstituted powders, and 3 g for unreconstituted powders. Add 25 mL laboratory water to flasks containing unreconstituted powders and mix until all of the powder dissolves. Add 1 mL of 6% taka-diastase to products containing starch. Allow taka-diastase to react with samples for at least 30 min before continuing with the extraction.

Note: Add 0.5 g milk protein such as calcium caseinate to nutritional products that do not contain any intact protein (i.e., infant elemental powders) and reconstitute or add water to the powder immediately before the extraction step.

(*2*) *Extraction*.—Add 30 mL 0.25 M sodium acetate buffer (pH 4.5) to each sample and swirl to mix. In a hood, add 1 mL freshly prepared 1% KCN to each sample and swirl to mix. Heat samples in a 105°C oven for at least 60 min, but for no more than 120 min. (Oven temperature will drop when the door is opened. Start timing when oven temperature returns to 105°C.) Remove samples from the oven and immediately cool in an ice bath. Dilute samples to volume with laboratory water. Mix well. Filter samples through Whatman 2V filter paper (www.whatman.com) into 125 mL Erlenmeyer flasks or equivalent glassware.

Note: If prepared samples are milky and contain very small insoluble particles, centrifuge samples and then transfer liquid layer to funnels lined with Whatman 2V filter paper.

Note: Do not heat samples to which 0.5 g milk protein has been added, but continue with the dilution and filtration steps.

(*3*) *Sample cleanup and concentration*.—For each sample, insert a 900 mg SPE cartridge onto the stopcock of the vacuum manifold and attach a 30 mL disposable syringe barrel to the top of each cartridge.

Note: Alltech C_8 and C_{18} cartridges can be used interchangeably. Condition each cartridge with at least 20 mL acetonitrile by allowing acetonitrile to pass by gravity through the cartridge and rinse each cartridge with at least 10 mL laboratory water.

Using volumetric pipets, transfer sample filtrates to cartridges using the guidelines in Table **2011.10D**. If the vitamin B_{12} concentration is unknown, use guidelines for RTF products containing 1–10 µg/L. If necessary apply enough vacuum so that the samples drip steadily through the cartridges. Sample filtrates should pass through the cartridges at a rate of no more than 120 drops/min. Discard eluent. After all of the sample filtrate has passed through the cartridge, rinse each cartridge with 5 mL

Table 2011.10D. Guidelines for loading sample filtrates onto SPE cartridges[a]

Vitamin B_{12} concentration in RTF product, µg/kg	Volume of filtrate loaded onto SPE cartridge, mL	Final dilution volume, mL
<1	80	5
1–10	70–80	10
11–20	50–60	10
21–50	20–40	10

[a] Do not load more than 60 mL adult and pediatric nutritionals onto an Alltech C_8 or C_{18} cartridge.

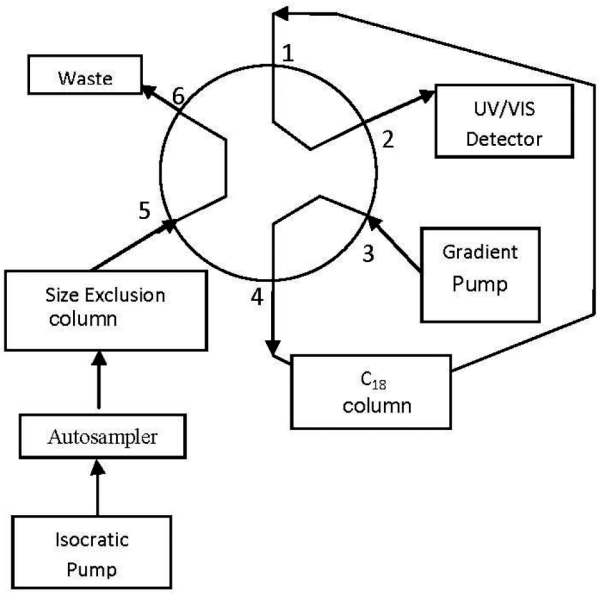

Figure 2011.10A. System setup and configuration: Configuration 1.

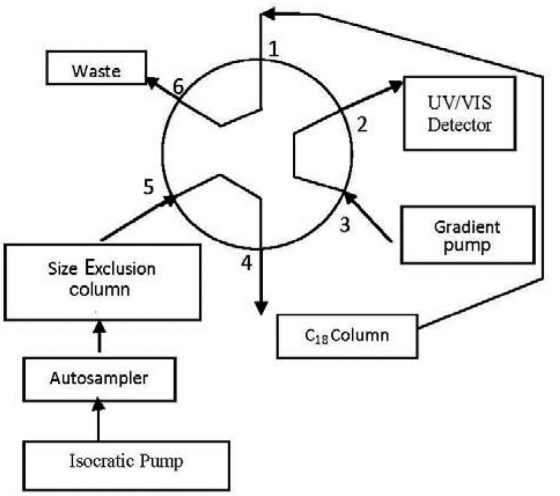

Figure 2011.10B. System setup and configuration: Configuration 2.

laboratory water and discard eluent. Air-dry each cartridge by pulling a vacuum until no more effluent is observed. Close each stopcock. Place a 5 or 10 mL volumetric flask under each cartridge. Add 4.4 mL 30% acetonitrile to each cartridge. Open each stopcock and elute vitamin B_{12} into the volumetric flasks.

(*4*) *Final dilution*.—For samples collected in 10 mL volumetric flasks, dilute to volume with water. For samples collected in 5 mL volumetric flasks, in a hood add 0.1 mL freshly prepared 0.4% KCN to each volumetric flask. Place prepared samples in a 95°C oven for at least 1.5 h, but for no more than 4 h. After at least 1.5 h, remove samples from the oven and cool to room temperature. Dilute to volume with laboratory water. Filter an aliquot of each standard and prepared sample through a 0.45 µm syringe filter into an autosampler vial.

(c) *HPLC analysis*.—(*1*) *System setup and configuration*.—*See* Figures **2011.10A** and **B** for configurations.

(*2*) *Instrument operation conditions*.—(*a*) *Run time*.—30–35 min.

(*b*) *Injection volume*.—900 µL to 2.0 mL.

(*c*) *System configuration*.—*See* Table **2011.10E**.

(*d*) *Isocratic pump*.—Mobile phase D: 2.5% acetonitrile. Flow rate: Adjust so that vitamin B_{12} elutes from the size-exclusion column between 10.5 and 14.5 min. Typical flow rates, 1.1–1.2 mL/min. *Note*: To determine an appropriate flow rate, connect the size-exclusion column directly to the UV-Vis detector and inject the high standard. Adjust flow rate as necessary so that vitamin B_{12} elutes between 10.5 and 14.5 min.

(*e*) *Gradient pump*.—Mobile phase compositions: mobile phase A, 0.4% TEA in laboratory water, pH 5–7; mobile phase B, 0.4% TEA and 25% acetonitrile in H_2O, pH 5–7; mobile phase C, 0.4% TEA and 75% acetonitrile in H_2O, pH 5–7. Determine an appropriate gradient to elute vitamin B_{12} in 23–30 min and resolve vitamin B_{12} from riboflavin using the information in Table **2011.10F**. (*See* Figure **2011.10C**.)

(*f*) *Gradient pump flow rate*.—1.0 mL/min.

(*g*) *Detector settings*.—Detection wavelengths and bandwidth, 550 and 10 nm, respectively.

(*3*) *HPLC of standards and samples*.—Make 3–4 injections of a working standard and verify the precision of those injections is ≤3%. If the system is working properly, inject a set of 3–6 working standards once, a set of 1–14 samples, and another set of 3–6 working standards. Every set of 1–14 samples should be bracketed by standards of appropriate concentration.

F. Calculations

(a) *Chromatography*.—Visually inspect each standard and sample chromatogram and verify that vitamin B_{12} is resolved from all other peaks in the chromatograms (Figures **2011.10D** and **E**).

(b) *Measurement of peak area*.—Peak areas are measured with a data system. Before calculating the vitamin B_{12} concentrations of samples, compare the vitamin B_{12} peak areas of the standards with the vitamin B_{12} peak areas of the samples and verify that the vitamin B_{12} peak areas of the samples are within the range of the vitamin B_{12} peak areas of the standards.

(c) *Calculation of standard concentration*.—

$$WS = S_w \times P \times A/200$$

where WS = working standard concentration in µg/L; S_w = amount of vitamin B_{12} standard weighed in mg; P = purity of USP reference standard in µg cyanocobalamin (vitamin B_{12})/mg of the standard; A = aliquot of vitamin B_{12} intermediate standard used (0.5, 1, 2, 3, 4, or 5) in mL; and 200 = dilution volume in mL.

(d) *Preparation of standard curves*.—(*1*) At each standard concentration, average the peak area of the standard injected at the beginning of a set of samples with the peak area of the standard injected at the end of the set of samples. Prepare a standard curve by performing linear least squares regression

Table 2011.10E. System configuration

Time, min	Valve configuration
0.00–10.5	Configuration 1
10.5–14.5	Configuration 2
14.5–30.0 to 33	Configuration 1

Table 2011.10F. RP column elution gradient

	Mobile phase, %		
Time, min	A	B	C
0.00	90	10	0
14.5	90	10	0
14.6	40–60[a]	60–40[a]	0
27.0–30	40–60[a]	60–40[a]	0
27.1–30.1	0	10	90
29.90–33.00	0	10	90

[a] Appropriate gradient conditions must be established with each column to adequately resolve vitamin B_{12} and riboflavin and to elute vitamin B_{12} between approximately 24 and 30 min. To establish appropriate gradient conditions with a new column, set the gradient composition at 14.6 and 27.0–30.0 min to the midpoint of the allowable range from the table above. Inject the resolution test solution and calculate the resolution (Rs) between vitamin B_{12} and riboflavin. Adjust the mobile phase composition between 14.6 and 27.0–30.0 min until Rs is >1.5. After vitamin B_{12} elutes from the C_{18} or phenyl column rinse the column with 90% mobile phase C for at least 2.8 min.

on concentration versus the average peak area of the working standards. A standard curve must have a correlation coefficient (r) of at least 0.999 to be considered acceptable for sample calculations. (*2*) At each working standard concentration, the peak areas of standards injected at the beginning and end of a set of samples should not increase or decrease by more than 10%.

(e) *Calculation of vitamin B_{12} concentrations in samples*.—The vitamin B_{12} concentration in each injected sample preparation is extrapolated from the vitamin B_{12} standard curve prepared as described above. The concentration of vitamin B_{12} in each product can then be calculated:

$$C_p = C_i \times D_1 \div ss \times D_2 \div V$$

where C_p = product concentration in µg/kg; C_i = vitamin B_{12} concentration of the injected sample preparation extrapolated from standard curve in µg/L; D_1 = volume of the first dilution in mL (100 mL); ss = sample size in g; D_2 = volume of the second (final) dilution in mL; and V = volume of filtrate loaded onto the cartridge in mL (2, 6).

Results and Discussion

Data from all participating laboratories are summarized in Tables 1 and 2. It should be noted that two of the participating laboratories (Laboratories 6 and 11) only received half of the study samples. It should also be noted that data for the second adult high-fat RTF replicate from four laboratories (Laboratories 1, 7, 9, and 11) had to be excluded because of sample mislabeling. Based on chromatographic profiles and physical appearance of the products, it was determined that some of the adult high-protein RTF samples were mislabeled as adult high-fat RTF samples. Chromatograms of the adult high-protein RTF samples, the adult high-fat RTF sample, and the mislabeled adult high-fat RTF sample are shown in Figures 1–4, illustrating the chromatographic differences between the two products. Although only five of the nine participating laboratories had duplicate results for the adult high-fat RTF because of mislabeling, AOAC recommended keeping the adult high-fat product in the study and calculating repeatability and reproducibility from the data that were available. As a result, repeatability was calculated from duplicate results generated at five laboratories, and reproducibility was calculated from the single and duplicate results from all nine laboratories.

Using the AOAC INTERNATIONAL Interlaboratory Study Blind (Unpaired) Replicates workbook, statistical outliers from one laboratory were identified for six of the 12 products analyzed. After removal of outliers, repeatability RSD_r ranged from 2.98 to 9.77% and met the SMPR ≤7% for eight of the 12 products analyzed. The RSD_R ranged from 3.54 to 19.5% and met the SMPR of ≤11% for seven of the 12 products analyzed. Although repeatability and reproducibility for several products exceeded the requirements in SMPR 2011.005, it was the majority opinion of the ERP that these results were

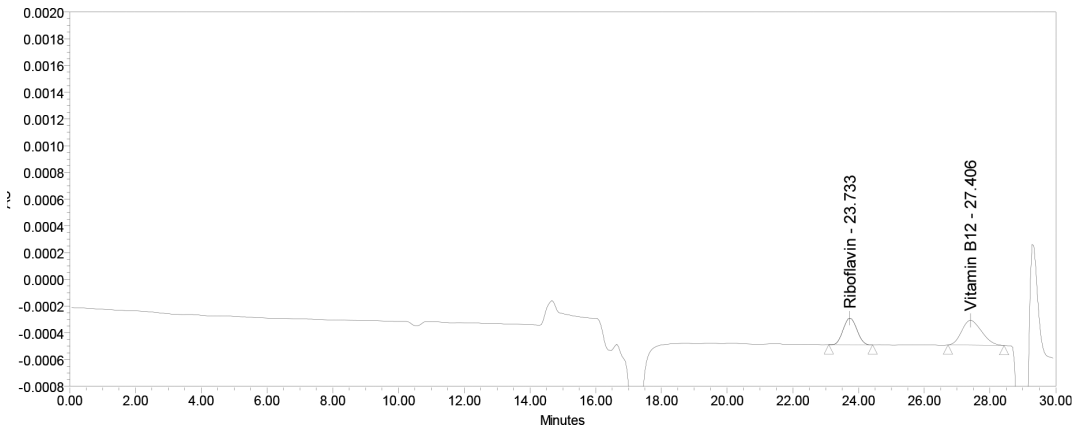

Figure 2011.10C. Example chromatogram showing riboflavin and vitamin B_{12} resolution.

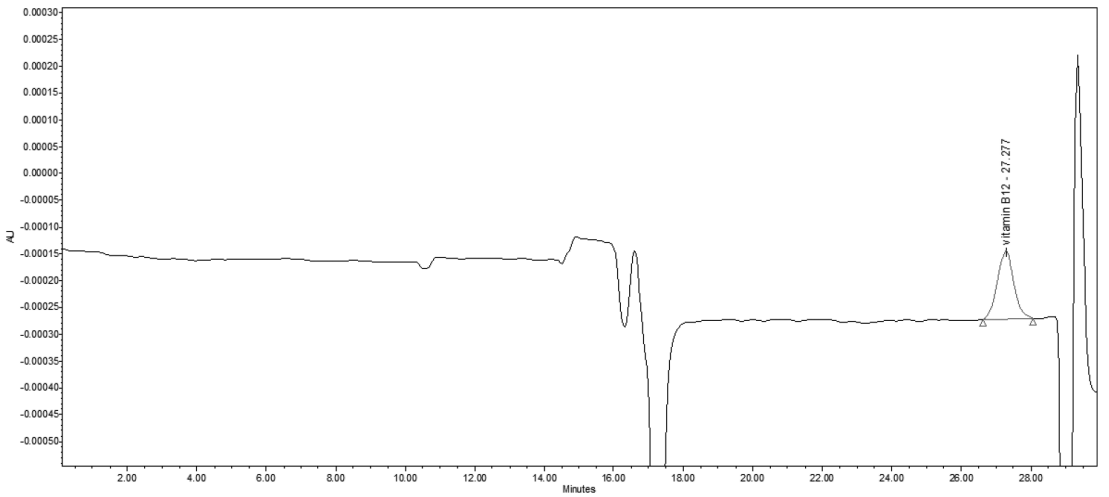

Figure 2011.10D. Typical standard chromatogram.

acceptable since repeatability only exceeded the requirement by 1–2%, and all of the products that exceeded the reproducibility requirement except one were near or below the minimum SMPR reproducibility level of 0.3 µg/100 g RTF liquid.

Several laboratories provided comments about the method. Some laboratories noted that they did not always know how much vitamin B_{12} was in the samples that they were testing, so it was hard to know how much filtrate to load onto the solid-phase extraction cartridge. Some laboratories had safety concerns about working with potassium cyanide, and another laboratory asked for a procedure to check SPE cartridge efficiency and vitamin B_{12} recovery. To address these concerns, a safety section and an SPE cartridge qualification procedure were added to the Final Action method.

Conclusions

AOAC Method **2011.10** was collaboratively studied by nine to 11 laboratories from eight different countries with a variety of infant, pediatric, and adult matrixes. Per the AOAC ERP, the method demonstrated acceptable repeatability and reproducibility and met the SPIFAN SMPR for the majority of product matrixes analyzed. Although repeatability and reproducibility for some product matrixes exceeded the requirements in SMPR 2011.005, it was the majority opinion of the ERP that these results were acceptable since repeatability only exceeded the requirement by 1–2%, and all of the products that exceeded the reproducibility requirement except one were near or below the minimum SMPR reproducibility level of 0.3 µg/100 g RTF liquid.

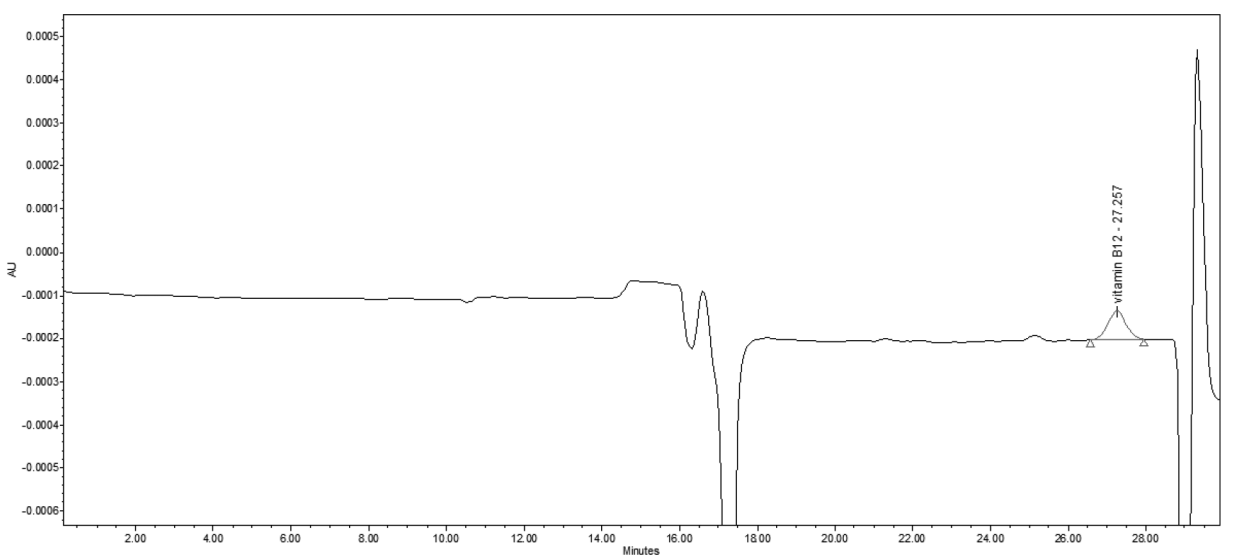

Figure 2011.10E. Typical sample chromatogram.

Table 1. Multilaboratory collaborative study data for vitamin B_{12}[a]

Lab No.	NIST 1849a		Adult milk powder		Infant partially hydrolyzed milk powder		Infant partially hydrolyzed soy powder		Adult low-fat powder		Child powder		Infant elemental powdered		Infant milk powder		Infant soy powder		Infant milk RTF		Adult high-fat RTF		Adult high-protein RTF	
1	42.1	45.4	0.306	0.308	0.360	0.359	0.212	0.222	0.687	0.680	0.934	0.963	0.532	0.516	0.227	0.230	0.415	0.431	0.290	0.295	1.53	1.19[b]	0.19	0.17
2	43.7	47.1	0.313	0.341	0.423	0.436	0.283	0.280	0.698	0.665	0.932	0.938	0.562	0.577	0.248	0.230	0.444	0.466	0.269	0.300	1.58	1.70	1.33	1.31
3	39.5	43.1	0.300	0.293	0.305	0.292	0.228	0.276	0.638	0.625	0.987	0.978	0.487	0.465	0.226	0.245	0.429	0.363	0.304	0.266	1.37	1.37	0.953	0.830
4	44.0	40.7	0.283	0.310	0.273	0.306	0.246	0.268	0.660	0.606	1.01	0.972	0.532	0.537	0.201	0.226	0.420	0.413	0.305	0.311	0.804[c]	0.683[c]	0.989	0.944
5	42.9	38.0	0.250	0.238	0.331	0.402	0.183	0.261	0.590	0.510	0.885[c]	0.767[c]	0.520[d]	0.670[d]	0.183	0.187	0.416	0.375	0.155	0.242	1.27	1.25	0.941	0.793
6	35.2[c]	19.9[c]	NA[e]	NA	0.305	0.312	0.173	0.155	0.627	0.570	NA	NA	0.466	0.491	NA	NA	0.271[c]	0.345[c]	NA	NA	NA	NA	NA	NA
7	44.8	54.9	0.304	0.250	0.395	0.355	0.236	0.234	0.671	0.609	0.950	0.899	0.584	0.633	0.248	0.243	0.436	0.420	0.225	0.252	1.33	0.872[b]	0.973	0.992
8	47.8	47.6	0.379	0.376	0.431	0.430	0.344	0.305	0.661	0.650	0.957	0.991	0.635	0.657	0.230	0.218	0.475	0.441	0.300	0.286	1.63	1.34	1.36	1.32
9	40.5	39.9	0.257	0.271	0.300	0.319	0.241	0.242	0.584	0.549	1.03	0.959	0.501	0.477	0.250	0.236	0.409	0.402	0.229	0.241	1.44	0.929[b]	1.13	0.893
10	42.6	40.2	0.363	0.279	0.471	0.487	0.317	0.284	0.686	0.757	0.965	1.01	0.564	0.556	0.330[f]	0.270[f]	0.469	0.477	0.296	0.327	1.79	1.57	1.33	1.26
11	43.3	45.0	0.267	0.319	0.463	0.456	NA	NA	NA	NA	NA	NA	NA	NA	0.244	0.221	0.438	0.421	NA	NA	1.59	0.932[b]	0.938	1.01

[a] Concentration in g/100 µg RTF liquid for all products except NIST 1849a; NIST 1849a concentration in µg/kg powder.

[b] Data excluded. Sample was mislabeled. Sample is really adult high-protein RTF liquid.

[c] Data excluded. Failed single Grubbs' test.

[d] Data excluded. Failed Cochran's test.

[e] NA = Not applicable. Laboratory did not receive sample.

[f] Data excluded. Failed double Grubbs' test.

Table 2. Multilaboratory collaborative study results for vitamin B_{12}

Sample type	Total No. labs[a]	Total No. replicates[b]	Mean, µg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat
Infant formula (NIST SRM 1849a)	11 (1)	22 (2)	43.70[c]	3.010	3.86	6.90	8.84	0.34
Infant formula powder soy-based	11 (1)	22 (2)	0.428	0.021	0.0305	4.85	7.13	0.20
Infant formula powder milk-based	10 (1)	20 (2)	0.227	0.011	0.0202	4.90	8.90	0.22
Infant formula RTF milk-based	9 (0)	18 (0)	0.272	0.026	0.0427	9.46	15.70	0.40
Infant formula powder partial hydrolyzed milk-based	11 (0)	22 (0)	0.373	0.020	0.0694	5.35	18.60	0.50
Infant formula powder partial hydrolyzed soy-based	10 (0)	20 (0)	0.250	0.024	0.0487	9.77	19.50	0.50
Adult nutritional powder milk-based	10 (0)	20 (0)	0.300	0.027	0.0416	8.99	13.80	0.36
Adult nutritional RTF high protein	10 (0)	20 (0)	1.080	0.073	0.1900	6.74	17.50	0.55
Child formula powder	9 (1)	18 (2)	0.967	0.029	0.0342	2.98	3.54	0.11
Adult nutritional RTF high fat	10 (1)	16 (2)	1.480	0.122	0.1710	8.23	11.50	0.38
Infant elemental powder	10 (1)	20 (2)	0.543	0.017	0.0603	3.11	11.10	0.32
Adult nutritional powder low fat	10 (0)	20 (0)	0.636	0.035	0.0587	5.47	9.23	0.27

[a] Number of laboratories submitting data (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Data reported as µg/kg powder.

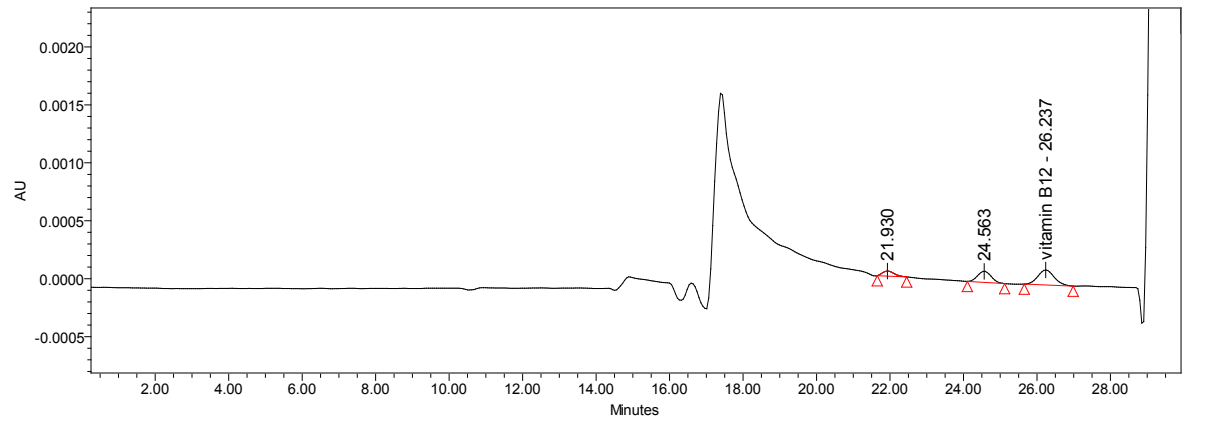

Figure 1. Chromatogram of blind duplicate labeled as LHTK069 (adult nutritional RTF high protein).

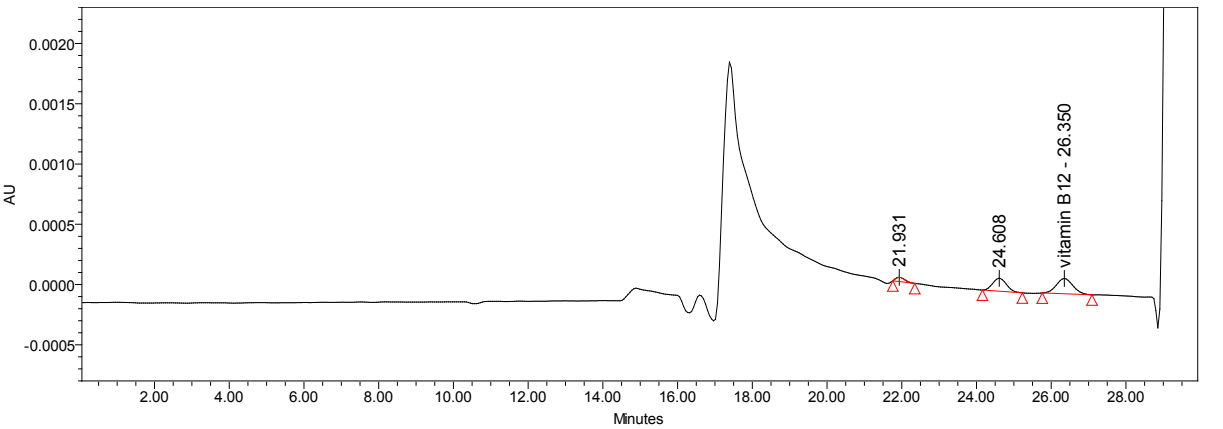

Figure 2. Chromatogram of blind duplicate labeled as LKAU043 (adult nutritional RTF high protein).

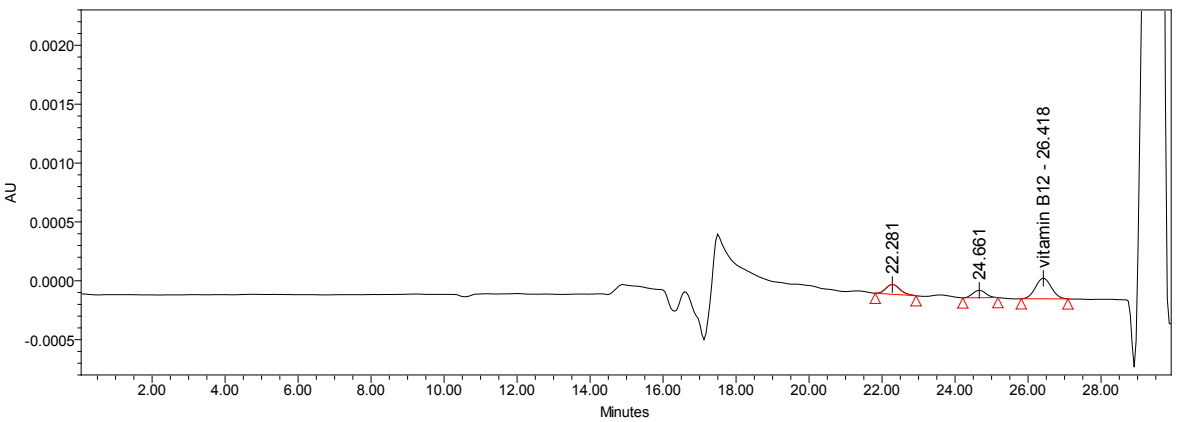

Figure 3. Chromatogram of blind duplicate labeled as VFJL09 (adult nutritional RTF high fat).

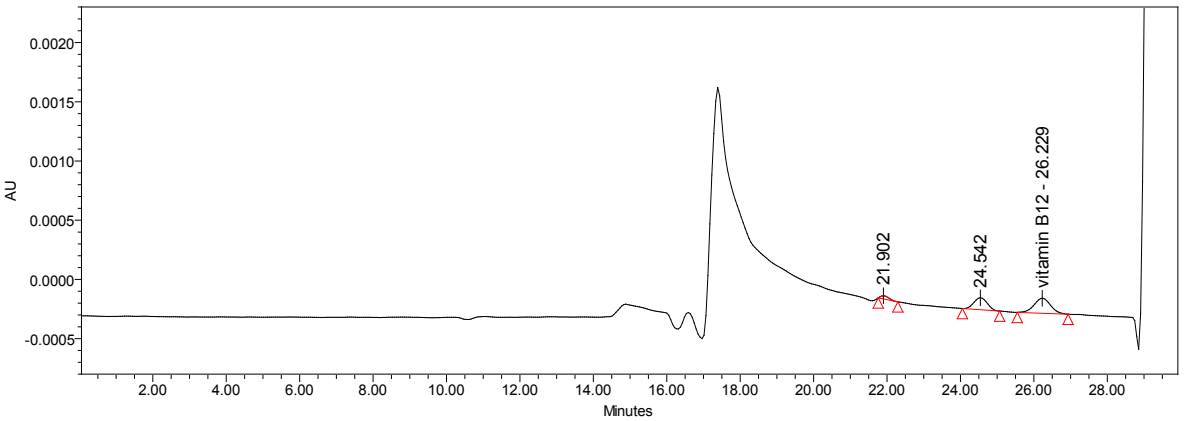

Figure 4. Chromatogram of blind duplicate labeled as YATV077 (adult nutritional RTF high fat)—mislabeled sample.

Recommendation

The multilaboratory collaborative study data were summarized and presented to the ERP in September 2014. After reviewing the data, the ERP voted to move AOAC **2011.10** to Final Action status, and the method was approved by the AOAC Official Methods Board as a Final Action method.

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Myo-Inositol in Infant, Pediatric, and Adult Formulas by Liquid Chromatography-Pulsed Amperometric Detection with Column Switching: Collaborative Study, Final Action 2011.18

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AOAC First Action Method 2011.18, Myo-Inositol (Free and Bound as Phosphatidylinositol) in Infant and Pediatric Formulas and Adult Nutritionals, was collaboratively studied. With this method free myo-inositol and phosphatidylinositol bound myo-inositol are extracted using two different sample preparation procedures, separated by ion chromatography using a combination of Dionex Carbo Pac PA1 and MA1 columns with column switching, and detected with pulsed amperometry using a gold electrode. Free myo-inositol is extracted from samples with dilute hydrochloric acid and water. Phosphatidylinositol is extracted from samples with chloroform and separated from other fats with silica SPE cartridges. Myo-inositol is then released from the glycerol backbone with concentrated acetic and hydrochloric acids at 120°C. During this collaborative study, nine laboratories from five different countries analyzed blind duplicates of nine infant and pediatric nutritional formulas for both free and phosphatidylinositol bound myo-inositol, and one additional laboratory only completed the free myo-inositol analyses. The method demonstrated acceptable repeatability and reproducibility and met the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) *Standard Method Performance Requirements* (SMPRs®) for free myo-inositol plus phosphatidylinositol bound myo-inositol for all the matrixes analyzed. SMPRs for repeatability were ≤5% RSD at myo-inositol concentrations of 2–68 mg/100 g ready-to-feed (RTF) liquid. SMPRs for reproducibility were ≤8% RSD in products with myo-inositol concentrations ranging from 2 to 68 mg/100 g RTF liquid. During this collaborative study, repeatability RSDs ranged from 0.51 to 3.22%, and RSDs ranged from 2.66 to 7.55% for free myo-inositol plus phosphatidylinositol bound myo-inositol.

Myo-inositol is a water-soluble hydroxylated, cyclic 6-carbon quasi-vitamin. Myo-inositol is regarded as a quasi-vitamin because it appears to satisfy the criteria of vitamin status for only a few species or only under certain conditions. It is the only one of the nine possible stereoisomeric forms of cyclohexitol with reported biological activity and is present in nearly all living cells. Myo-inositol occurs in foods mainly as free myo-inositol, phytic acid, and inositol-containing phospholipids. Plants seeds are the richest sources of myo-inositol, but it is present predominantly as phytic acid which is a poor source of myo-inositol because most mammals have little or no intestinal phytase activity. Myo-inositol is present in animal products such as milk, eggs, and meat primarily as free myo-inositol and phosphatidylinositol (1). Phosphatidylinositol is also present in soy flour and lecithin. Although the predominant inositol phosphate in foods is myo-inositol hexakisphosphate or phytic acid ($InsP_6$), other inositol phosphates such as myo-inositol tris- ($InsP_3$), tetrakis- ($InsP_4$), and pentakis- ($InsP_5$) phosphate may be present in some foods in relatively minor amounts (2).

The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) defined myo-inositol as free myo-inositol and phosphatidylinositol, excluding methyl ethers, glycosides, phosphorylated forms, and phytate. Nutritional products are fortified with free myo-inositol, but they may contain some inherent free, phosphatidylinositol bound myo-inositol, inositol phosphates, and phytate. The myo-inositol SPIFAN working group developed *Standard Method Performance Requirements* (SMPRs®) that were then approved by SPIFAN (3).

A reference method capable of determining only free myo-inositol and myo-inositol bound as phosphatidylinositol in infant, adult, and pediatric formula powders; ready-to-feed (RTF) liquids; and liquid concentrates was needed since there were no myo-inositol reference methods. Traditional microbiological methods are not precise and accurate enough to meet the nutrient specification requirements of infant,

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The method was approved by the AOAC *Official Methods Board* as Final Action. *See* "Standards News," (2014) *Inside Laboratory Management*, November/December 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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pediatric, and adult nutritionals, and none of the published myo-inositol methods will only determine both free myo-inositol and myo-inositol bound as phosphatidylinositol.

In 2011 and 2012, the AOAC Expert Review Panel (ERP) on SPIFAN Nutrient Methods granted First Action status to two inositol methods, AOAC **2011.18** (4) and AOAC **2012.12** (5). Both of these methods use ion chromatography and pulsed amperometric detection with a gold electrode, but they differ in their sample preparation procedures and chromatographic separations. Single-laboratory validations (SLVs) were completed with both of these methods, and in March 2013 AOAC **2011.18** was selected by the ERP for further evaluation with a multilaboratory collaborative study to determine method reproducibility (4, 6).

Consistent with the requirements of the SMPRs, the method chosen by the ERP for a multilaboratory collaborative study (AOAC **2011.18**) determines only free and phosphatidylinositol bound myo-inositol. With this method, free myo-inositol and phosphatidylinositol bound myo-inositol are extracted using two different sample preparation procedures, separated by ion chromatography using a combination of Dionex PA1 and MA1 columns with column switching, and detected with pulsed amperometry using a gold electrode. Free myo-inositol is extracted from samples with dilute hydrochloric acid and water. Phosphatidylinositol is extracted from samples with chloroform and separated from other fats with silica SPE cartridges. Myo-inositol is then released from the glycerol backbone with concentrated acetic and hydrochloric acids at 120°C.

Multilaboratory Collaborative Study

Initially 15 laboratories expressed interest in completing the multilaboratory collaborative study, but only 10 laboratories were able to participate. The 10 participating laboratories were located in five different countries. The remaining five laboratories were not able to participate because of time and resource constraints and issues with the importation of samples into their countries. One of the participating laboratories did not receive the liquid products, and one laboratory was only able to complete the free myo-inositol testing.

Before actual multilaboratory collaborative study samples were analyzed, each participating laboratory was asked to analyze a practice sample, Standard Reference Material (SRM) 1849a, in duplicate in order to identify and resolve any testing issues that they may have had executing the method. During the analysis of the practice sample for phosphatidylinositol bound myo-inositol, it was discovered that there were significant differences between the ovens used by the study participants. Even though all oven temperatures were set at 110°C, it appeared that in some laboratories samples did not receive enough heat during the hydrolysis step to efficiently release bound myo-inositol. In all cases when laboratories were asked to increase oven temperatures 10–20°C, myo-inositol recoveries improved. After approval of the practice sample results by the Study Directors, laboratories began testing the study samples.

Each participating laboratory received blind duplicates of nine SPIFAN matrixes fortified with myo-inositol or with significant levels of inherent myo-inositol. The SPIFAN matrixes analyzed included SRM 1849a, an infant formula partially hydrolyzed milk based powder, an infant formula partially hydrolyzed soy-based powder, a child formula powder, an infant elemental powder, an infant formula milk-based powder, an infant formula soy-based powder, an infant formula milk-based RTF, and an unfortified infant formula milk-based RTF.

Per SPIFAN requirements, participants were asked to reconstitute all powders prior to analysis. SRM 1849a 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 of powder in 200 mL laboratory water. For free myo-inositol analyses, participants were asked to analyze all 18 samples on one day, and for phosphatidylinositol bound myo-inositol analyses participants were asked to split the 18 samples into two groups of 10 and eight according to the data reporting sheets in the study protocol and to test each group on a separate day. Although the original AOAC First Action method **2011.18** published in the *Journal of AOAC INTERNATIONAL* (4) included a step to dilute each sample with water before adding 50% sodium hydroxide after sample hydrolysis, this step was accidentally omitted from the Official First Action method on the AOAC website and the original AOAC **2011.18** multilaboratory study protocol. This error was discovered after one of the participating laboratories reported a more vigorous than expected reaction when 50% sodium hydroxide was added to the hydrolyzed sample. The protocol was corrected, and the revised protocol was sent to all study participants. It should also be noted that the original method validation data published in the First Action **2011.18** manuscript (4) were generated using direct powder weights.

Upon completion of the sample analyses, participating laboratories were asked to send all of their data to the Study Directors. This included all standard and sample chromatograms for the instrument check, practice and test sample analyses, standard curve information, calculations, and completed Reporting of Analysis Forms with dilution and sample weights. Participants were also asked to report any deviations to the method and any relevant comments based on their experiences with the method.

All data were statistically analyzed according to AOAC INTERNATIONAL guidelines to determine overall mean, repeatability SD (s_r), repeatability RSD (RSD_r), reproducibility SD (s_R), reproducibility RSD (RSD_R), and Horwitz ratio (HorRat; 7). Cochran ($P = 0.025$, one-tail) and Grubbs' (single and double, $P = 0.025$, two-tail) tests were used to determine statistical outliers.

Myo-inositol SPIFAN SMPRs for repeatability were ≤5% RSD at myo-inositol concentrations of 2–68 mg/100 g RTF liquid. Myo-inositol SPIFAN SMPRs for reproducibility were ≤8% RSD in products with myo-inositol concentrations ranging from 2 to 68 mg/100 g of RTF liquid.

Method

A few minor modifications were made to AOAC Official First Action **2011.18** before it was sent to the multilaboratory collaborative study participants. These changes included updating the pulsed amperometric detector (PAD) program and increasing the sodium hydroxide concentration from 750 mM to 1 M. The PAD program listed in AOAC **2011.18**, First Action, was the waveform that was used when the method was originally developed over 15 years ago using equipment that is now obsolete. After First Action status was granted to AOAC **2011.18** and before completion of the SPIFAN SLV and multilaboratory collaborative study, the obsolete PAD was replaced with a newer model, and the waveform listed in AOAC **2011.18** was updated based on the recommendations of the instrument manufacturer. The updated waveform was added to the multilaboratory collaborative study protocol and to AOAC **2011.18**, Final Action, and the previous waveform was removed. It should be noted that whenever changes are made to the PAD, the accuracy of the detector waveform should be verified by analyzing an SRM and performing free myo-inositol spike recovery experiments with an infant or adult formula containing hydrolyzed protein.

It should also be noted that although the multilaboratory study protocol specified using a standard (not disposable) gold electrode, some participating laboratories used disposable gold working electrodes. The instruction to not use disposable electrodes was removed from the AOAC **2011.18** Final Action method.

After completion of the multilaboratory collaborative study, the modifications noted above were incorporated in the Final Action method along with a few additional modifications based on study results and feedback from study participants and the ERP.

For this reason, the method described below is slightly different than the Official Final Action method currently posted on the AOAC website.

AOAC Official Method 2011.18
Myo-Inositol (Free and Bound as Phosphatidylinositol) in Infant and Pediatric Formula and Adult Nutritionals
Liquid Chromatography/Pulsed Amperometry with Column Switching
First Action 2011
Final Action 2014

ISO–AOAC METHOD

The LC method with electrochemical (pulsed amperometry) detection (PAD) allows for the quantitation of myo-inositol in infant, pediatric, and adult nutritional formulas. The concentration of myo-inositol is calculated by comparison with standards of known concentration. Myo-inositol, as defined by AOAC Standard Method Performance Requirement (SMPR®) 2011.007 (free and bound as phosphatidylinositol), can be calculated by adding the free myo-inositol and myo-inositol bound as phosphatidylinositol data. The method was validated for the quantitation of free myo-inositol and myo-inositol from phosphatidylinositol in infant, pediatric, and adult nutritionals. Repeatability was determined from duplicate analyses performed on multiple days. Accuracy was determined from spike recovery experiments (free myo-inositol and myo-inositol from phosphatidylinositol). Instrument LODs and LOQs were determined statistically from injections of low-level standards and by spiking samples with low levels of free myo-inositol. *See* Tables **2011.18 A–C** for results of single- and multilaboratory studies supporting acceptance of the method.

Caution: Refer to Material Safety Data Sheets (MSDS) of chemicals prior to use and follow safe handling procedures and the suggested personal protective equipment. Chloroform is a hazardous chemical and should be handled in a fume hood. Perform the phosphatidylinositol bound myo-inositol extraction and SPE sample cleanup procedure in a fume hood.

A. Apparatus

(a) *Analytical balance.*—Minimum weighing capacity of at least 0.0001 g.

(b) *Centrifuge.*

(c) *Desiccator.*

(d) *N-evap.*—With water bath (Organomation Associates, Inc., Berlin, MA) or equivalent.

(e) *Oven.*—Capable of maintaining 120°C.

(f) *pH meter.*—With pH 4 and 7 buffers.

(g) *Stir plate.*—Multiposition with stir bars.

(h) *Vacuum manifold.*

(i) *Vortex mixer.*

(j) *HPLC system.*—Corrosion-resistant components, including an autosampler, two isocratic pumps, 6-port switching valve, pulsed amperometry detector with a gold electrode and polyether ether ketone or Teflon 0.007–.01 in. id tubing. Autosampler capable of injecting 20 µL.

(k) *Columns.*—Dionex CarboPac MA1 (4 × 250 mm) P/N 44066, MA1 (4 × 50 mm) P/N 44067, and PA1 (4 × 50 mm) P/N 43096, or equivalent (www.thermofisher.com/dionex/).

B. Materials

(a) *Beakers.*—Assorted sizes.

(b) *Centrifuge tubes.*—50 mL with Teflon-coated caps.

(c) *Syringe filters.*—Nylon, 0.45 and 0.2 µm.

(d) *Filter paper.*—Whatman 2 V or equivalent (www.whatman.com).

(e) *Erlenmeyer flasks.*—50 or 125 mL or equivalent.

(f) *Volumetric flasks.*—Assorted sizes.

(g) *Funnels.*—Suitable for use with filter paper.

(h) *Pipets.*—Volumetric (Class A); assorted sizes.

(i) *SPE cartridges.*—Silica, 1 g (J.T. Baker Inc., Phillipsburg, NJ; P/N 7086-07, www.avantormaterials.com) or equivalent.

(j) *Syringes.*—1 mL disposable and 25 mL gas-tight glass with 4 in. stainless steel needles.

Table 2011.18A. SLV repeatability precision data for myo-inositol

Sample type	No. of replicates (duplicates on multiple days)	Mean, mg/100 g RTF	SD_r	RSD_r, %
Free myo-inositol				
Infant formula (NIST SRM 1849a)	14	415[a]	8.03	2.00
Infant formula powder soy-based	14	4.19	0.0910	2.17
Infant formula powder milk-based	14	4.21	0.0977	2.32
Infant formula RTF milk-based	14	7.19	0.250	3.48
Infant formula powder partial hydrolyzed milk-based	14	3.38	0.0997	2.95
Infant formula powder partial hydrolyzed soy-based	14	3.10	0.0626	2.02
Infant elemental powder	14	4.85	0.148	3.06
Child formula powder	14	5.04	0.112	2.22
Infant formula RTF milk-based, unfortified	14	3.17	0.0466	1.47
Infant elemental powder, unfortified	12	1.74	0.0329	1.89
Child formula powder, spiked	12	1.94	0.0477	2.46
Adult nutritional RTF high protein, spiked	12	61.4	1.87	3.05
Myo-inositol bound as phosphatidylinositol				
Infant formula (NIST SRM 1849a)	12	10.6[a]	0.536	5.05
Infant formula powder soy-based	12	2.48	0.0595	2.40
Infant formula powder partial hydrolyzed milk-based	12	0.244	0.00976	4.00
Infant formula powder partial hydrolyzed soy-based	12	1.98	0.0664	3.36
Child formula powder	12	0.443	0.0196	4.43
Adult nutritional powder, milk protein-based	12	1.43	0.067	4.69
Free myo-inositol plus myo-inositol bound as phosphatidylinositol				
Infant formula (NIST SRM 1849a)	12	426[a]	8.35	1.96
Infant formula powder soy-based	12	6.67	0.109	1.63
Infant formula powder partial hydrolyzed milk-based	12	3.63	0.100	2.76
Infant formula powder partial hydrolyzed soy-based	12	5.08	0.0914	1.80
Child formula powder	12	5.48	0.113	2.07

[a] Results in mg/kg powder.

C. Chemicals and Solvents

(a) *Acetic acid.*—Glacial, ACS.

(b) *Chloroform.*—High-purity, HPLC grade.

(c) *Diethyl ether.*—Anhydrous, HPLC grade.

(d) *Drierite (desiccant).*—Anhydrous calcium sulfate, 8 mesh.

(e) *Helium.*—Zero grade or equivalent.

(f) *Hexane.*—HPLC grade.

(g) *Hydrochloric acid.*—Concentrated (36–38%), ACS.

(h) *Laboratory water.*—≥15 MΩ·cm.

(i) *Metaphosphoric acid.*—ACS.

(j) *Methanol.*—HPLC grade.

(k) *Myo-inositol.*—USP (Rockville, MD) reference standard, official lot; store desiccated. *See* standard label for purity.

(l) *Sodium chloride.*—ACS.

(m) *Sodium hydroxide.*—50% (w/w), low carbonate form.

D. Preparation of Reagents and Standard Solutions

All solutions can be scaled up or down for convenience provided good laboratory practices are observed. Solutions can be stored at 2–30°C in tight, inert containers unless otherwise noted.

(a) *Myo-inositol stock standard solution (approximately 2000 mg/L).*—Accurately weigh approximately 0.100 g myo-inositol and quantitatively transfer to a 50 mL volumetric flask. Dilute to volume with water. Mix well. Store refrigerated. Expiration: 3 months.

(b) *Myo-inositol intermediate standard solution (approximately 200 mg/L).*—Dilute 10.0 mL stock standard to 100 mL with laboratory water and mix well. Discard after use.

(c) *Myo-inositol working standard solutions (approximately 4, 2, 1, 0.5, 0.2, and 0.05 mg/L).*—Dilute 2.0, 1.0, and 0.5 mL myo-inositol intermediate standard to 100 mL with laboratory water (4, 2, and 1 mg/L). Dilute 0.5 mL myo-inositol intermediate standard to 200 mL with laboratory water (0.5 mg/L). Dilute 4 and 1 mL 0.5 mg/L myo-inositol working standard to 10 mL with laboratory water (0.2 and 0.05 mg/L). Expiration: 2 weeks.

(d) *Hydrochloric acid, 0.5%.*—Add 1.25 mL concentrated hydrochloric acid to approximately 200 mL water in a 250 mL volumetric flask. Dilute to volume with water and mix well. Expiration: 6 months.

(e) *Sodium chloride, 1 M.*—Dissolve 5.8 g sodium chloride and dilute to 100 mL with water. Expiration: 1 month.

(f) *Sodium hydroxide, 0.12% or 30 mM (Pump 1).*—Quickly weigh 4.8 (±0.1) g 50% sodium hydroxide into a 2000 mL volumetric flask containing approximately 1900 mL water.

Table 2011.18B. SLV accuracy (spike recovery) data for myo-inositol

Sample type	No. of replicates (duplicates on multiple days)	Native level, mg/100 g RTF	Spike level, mg/100 g RTF	Recovery, %	RSD, %
Free myo-inositol					
Child formula powder, unfortified	12	0.0426	1.89	101	2.78
Infant formula powder soy-based	12	4.18	14.9	98.2	2.47
Infant formula powder partial hydrolyzed milk-based	12	3.35	15.2	102	3.00
Infant elemental powder, unfortified	12	0.00	1.74	93.9	3.00
Infant elemental powder, unfortified	6	0.00	1.09	93.2	1.54
Infant elemental powder, unfortified	6	0.00	0.390	90.2	3.14
Adult nutritional powder milk-based	12	0.409	65.0	101	2.64
Adult nutritional RTF high protein	12	0.042	61.4	96.3	3.27
Myo-inositol bound as phosphatidylinositol					
Infant formula (NIST SRM 1849a)	6	11.1[a]	7.3	77.1	13.2
Infant elemental powder, unfortified	6	0.00	0.294	87.3	6.88
Child formula powder	6	0.443	0.340	90.0	15.7
Infant formula powder soy-based	6	2.48	3.22	81.9	0.21
Infant formula powder partial hydrolyzed milk-based	6	0.247	0.152	79.9	7.51
Infant formula powder partial hydrolyzed soy-based	6	1.94	2.47	72.8	5.18
Adult nutritional powder milk-based	6	1.43	1.54	75.7	3.23

[a] Results in mg/kg powder.

(*Note*: It is important that the sodium hydroxide does not absorb carbon dioxide from the air.) Swirl to mix well. Dilute to volume with water and mix well. Expiration: 1 month.

(g) *Sodium hydroxide, 4.0% or 1 M (Pump 2).*—Quickly weigh 160 (±3) g 50% sodium hydroxide into a 2000 mL volumetric flask containing approximately 1900 mL water. (*Note*: It is important that the sodium hydroxide does not absorb carbon dioxide from the air.) Swirl to mix well. Dilute to volume with water and mix well. Expiration: 1 month.

(h) *6% Metaphosphoric acid.*—Weigh 6.0 g metaphosphoric acid into a 100 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Store refrigerated. Expiration: 1 week.

(i) *Phosphatidylinositol extraction solutions.*—Prepare fresh on day of use.

(*1*) *Chloroform–methanol (2 + 1).*—Mix 60 mL chloroform and 30 mL methanol.

(*2*) *Hexane–diethyl ether (80 + 20).*—Mix 80 mL hexane and 20 mL diethyl ether.

(*3*) *Hexane–diethyl ether (50 + 50).*—Mix 50 mL hexane and 50 mL diethyl ether.

(*4*) *Methanol–chloroform–water (75 + 15 + 10).*—Mix 75 mL methanol, 15 mL chloroform, and 10 mL water.

E. Sample Preparation and Extraction

(a) *Sample preparation for free myo-inositol determinations.*—Prepared samples that are constantly stored at 1–8°C in closed containers are stable for up to 5 days. After 5 days, samples must be prepared again.

Thoroughly mix or stir products prior to sampling. For liquid products, accurately weigh 0.5 to 5 g (±10%) product into a 100 mL volumetric flask and record the weight to the nearest 0.0001 g. For powdered products that do not require reconstitution, accurately weigh 0.25–1.5 g powder into a 100 mL volumetric flask and record the weight to the nearest 0.0001 g. Add approximately 10 to 15 mL laboratory water to the volumetric flask and swirl or stir to completely dissolve the powder. For powdered products that are not homogeneous at the subgram level, reconstitute following the product label instructions and accurately weigh 0.5 to 5 g reconstituted product into a 100 mL volumetric flask. Record the weight to the nearest 0.0001 g. Add enough 0.5% hydrochloric acid to each sample to adjust the sample pH to 4.5 ± 0.2 and swirl to mix.

Allow the samples to react with 0.5% hydrochloric acid for a minimum of 2 min and then dilute to volume with laboratory water. Mix well. Filter samples through Whatman 2V filter paper into 125 mL Erlenmeyer flasks or appropriate glassware. (*Note*: Although some samples will filter cloudy, the filtrates can still be used.) Filter an aliquot of sample filtrate through a 0.45 µm syringe filter into an autosampler vial.

(b) *Sample preparation for myo-inositol bound as phosphatidylinositol determinations.*—(*1*) *Extraction.*—Weigh 4 g (±10%) liquid or reconstituted powder product or 1 g (±10%) homogeneous powder into a 50 mL centrifuge tube and record the weight to the nearest 0.0001 g. Add 4 mL laboratory water to 1 g homogeneous powder samples. In a fume hood, add 10 mL methanol and stir for at least 20 min or vortex for at least 1 min and allow samples to set for at least 20 min. Add 20 mL chloroform and stir for at least 5 min or vortex for at least 1 min and allow samples to set for at least 5 min. If large clumps form when chloroform is added, cap tube and shake well for at least 1 min to mix sample. Add 5 mL 6% metaphosphoric acid and

Table 2011.18C. Interlaboratory study results for myo-inositol with all MLT data included and outliers removed

Sample type	Total No. labs	Total No. replicates	Mean, mg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat[a]
Free myo-inositol								
Infant formula (NIST SRM 1849a)	10	22	4.12[b]	11.3	11.4	2.75	2.77	0.43
Infant formula powder soy-based	10	22	4.22	0.127	0.305	3.03	7.26	0.80
Infant formula powder milk-based	10	20	4.26	0.168	0.232	3.95	5.43	0.60
Infant formula RTF milk-based	9	20	7.17	0.095	0.207	1.33	2.89	0.34
Infant formula powder partial hydrolyzed milk-based	10	22	3.65	0.035	0.412	0.97	11.4	1.22
Infant formula powder partial hydrolyzed soy-based	10	22	3.11	0.0899	0.389	2.92	12.6	1.32
Child formula powder	10	22	5.10	0.185	0.246	3.61	4.81	0.54
Infant elemental powder	10	22	5.10	0.227	0.318	4.45	6.24	0.71
Infant formula RTF milk-based, unfortified	9	20	3.17	0.0582	0.0910	1.84	2.87	0.30
Myo-inositol bound as phosphatidylinositol								
Infant formula (NIST SRM 1849a)	9	20	9.51[b]	1.82	2.62	18.7	26.8	2.36
Infant formula powder soy-based	9	20	2.10	0.150	0.501	6.94	23.2	2.30
Infant formula powder milk-based	9	18	0.667	0.0261	0.172	3.92	25.9	2.15
Infant formula RTF milk-based	8	18	0.348	0.0301	0.0909	8.36	25.2	1.91
Infant formula powder partial hydrolyzed milk-based	9	20	0.214	0.0103	0.0576	4.72	26.4	1.86
Infant formula powder partial hydrolyzed soy-based	9	20	1.64	0.0936	0.358	5.53	21.1	2.02
Child formula powder	9	20	0.328	0.0234	0.0878	6.89	25.8	1.94
Infant elemental powder	9	20	0.00	0.00	0.00	0.00	0.00	0.00
Infant formula RTF milk-based, unfortified	8	18	0.305	0.0244	0.0850	7.71	26.9	2.00
Free myo-inositol plus myo-inositol bound as phosphatidylinositol								
Infant formula (NIST SRM 1849a)	9	20	422[b]	11.9	11.9	2.83	2.83	0.44
Infant formula powder soy-based	9	20	6.27	0.147	0.446	2.32	7.05	0.82
Infant formula powder milk-based	9	18	4.92	0.184	0.314	3.74	6.38	0.72
Infant formula RTF milk-based	8	18	7.50	0.106	0.218	1.41	2.90	0.35
Infant formula powder partial hydrolyzed milk-based	9	20	3.84	0.035	0.426	0.91	11.2	1.21
Infant formula powder partial hydrolyzed soy-based	9	20	4.71	0.152	0.357	3.22	7.55	0.84
Child formula powder	9	20	5.42	0.203	0.307	3.73	5.63	0.64
Infant elemental powder	9	20	5.08	0.237	0.324	4.67	6.40	0.72
Infant formula RTF milk-based, unfortified	8	18	3.46	0.0659	0.128	1.90	3.70	0.39

[a] HorRat = RSD/PRSD; PRSD = $2C^{-0.15}$ (C = concentration by mass fraction).

[b] Results in mg/kg powder.

1 mL 1 M NaCl and mix well. Centrifuge until layers separate. Using a 25 mL glass tight syringe with a 4 in. stainless steel needle, transfer the bottom chloroform layer to a clean 50 mL centrifuge tube and evaporate the chloroform with nitrogen in a 60°C water bath.

(*2*) *Sample cleanup*.—In a fume hood, condition a 1 g silica SPE cartridge with 6 mL hexane. Dissolve residue in bottom of centrifuge tube in 1 mL chloroform–methanol (2 + 1). Quantitatively transfer dissolved residue to the conditioned silica SPE cartridge. Rinse 50 mL centrifuge tube with 3 mL hexane–diethyl ether (80 + 20) and then transfer to the SPE cartridge. Discard the eluent. Rinse 50 mL centrifuge tube with 3 mL hexane–diethyl ether (50 + 50) and then transfer to the SPE cartridge. Collect eluent in a clean 50 mL centrifuge tube. Rinse 50 mL centrifuge tube with 4 mL methanol and then transfer to the SPE cartridge. Collect eluent in the same

Table 2011.18D. Instrument operating conditions

Pump 1 pressure limit	2000 psi
Pump 1 mobile phase	0.12% (30 mM) NaOH
Pump 1 flow rate	0.4 mL/min
Pump 2 pressure limit	2000 psi
Pump 2 mobile phase	4% (1 M) NaOH
Pump 2 flow rate	0.4 mL/min
Injection volume	20 µL
Myo-inositol retention time	11–13 min
Run time	25 min
Switching valve configuration time table	
t, min	Configuration
0.00	1 (*see* Figure **2011.18A**)
1.50	2 (*see* Figure **2011.18B**)
13.50	1 (*see* Figure **2011.18A**)

Table 2011.18E. PAD settings with gold electrode

Analog range	1 uC	
Detector program: Dionex ICS3000 or ICS 5000	t, s	E, V
	0.0	+0.10
	0.20	+0.10
	0.40	+0.10
	0.41	–2.00
	0.42	–2.00
	0.43	+0.60
	0.44	–0.10
	0.50	–0.10
Integration period	0.20–0.40 s	

50 mL centrifuge tube. Rinse 50 mL centrifuge tube with 4 mL methanol–chloroform–water (75 + 15 + 10) and transfer to the SPE cartridge. Collect eluent in the same 50 mL centrifuge tube. Evaporate eluents collected from SPE cartridge with nitrogen in a 60°C water bath.

(*3*) *Hydrolysis*.—In a fume hood, add 40 µL glacial acetic acid and 2 mL concentrated hydrochloric acid to the residue in the centrifuge tube from the sample cleanup step. Tightly cap tube. Heat in a 120°C oven for 2 h. Cool. Add about 10 mL laboratory water and swirl to mix. Add 1.25 mL 50% (w/w) sodium hydroxide. Transfer sample to a 50 mL volumetric flask and dilute to volume with water. Filter an aliquot of sample filtrate through a 0.45 µm syringe filter into an autosampler vial.

(c) *HPLC analysis*.—(*1*) *See* Tables **2011.18D** and **2011.18E** for instrument operating conditions and PAD settings, respectively.

(*2*) *Instrument startup*.—The HPLC system should be located in an area where temperature fluctuations will be minimal throughout the run.

Prepare mobile phases. If necessary, helium sparge mobile phases and/or pressurize mobile phase reservoirs. If necessary, clean and polish the gold working electrode. Turn on the detector and pump mobile phase through the columns at a flow rate of 0.40 mL/min for at least 30 min to equilibrate the system. Verify that the detector is stable before beginning an analysis. Inject 20 µL of the most concentrated standard at least five times and note the peak areas or heights. If the system is equilibrated, the RSD of the peak areas or heights of the last three standard injections should be ≤2.0%.

(*3*) *Standard and sample analysis*.—Once the system has equilibrated, inject one standard at each concentration. After a set of standards has been injected, a control sample and up to 14 samples can be injected before another set of standards should be injected.

(*4*) *System shutdown*.—After all samples and standards have been analyzed, inject 20 µL of water to clean out the autosampler needle and tubing. Store the analytical columns in mobile phase [0.12% (30 mM) sodium hydroxide]. Turn off the electrochemical cell. Flush the pump heads with water to remove sodium hydroxide.

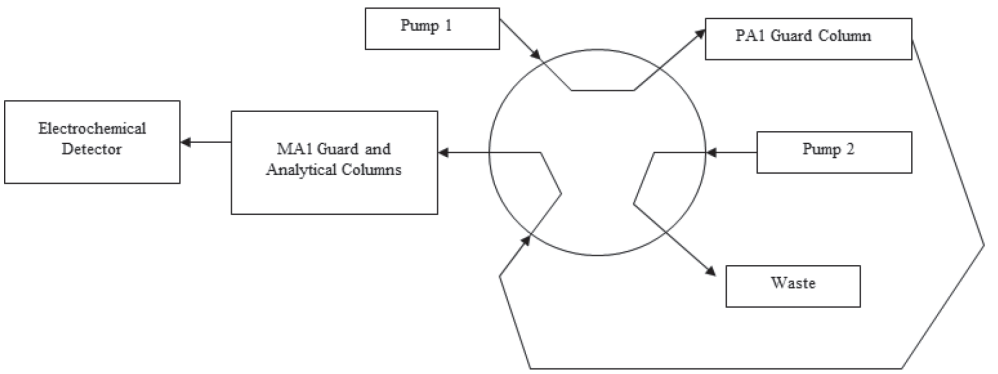

Figure 2011.18A. Switching valve configuration 1.

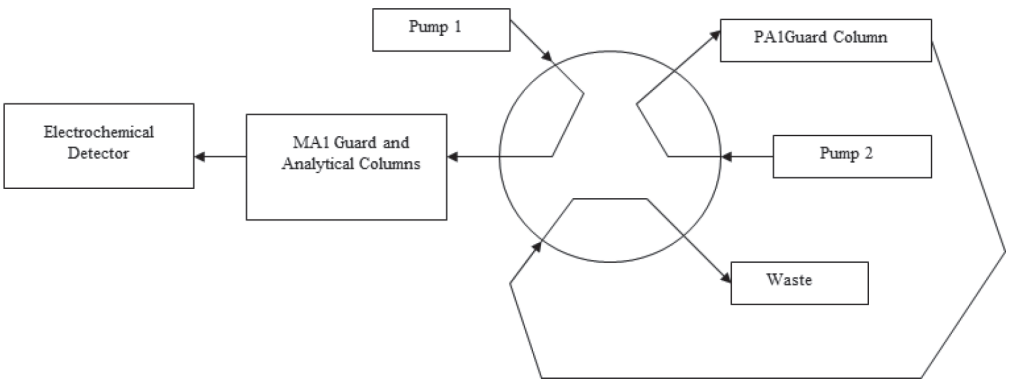

Figure 2011.18B. Switching valve configuration 2.

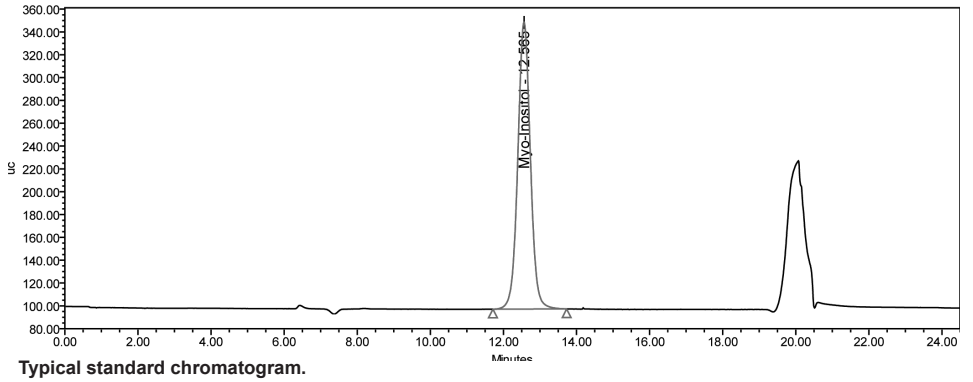

Figure 2011.18C. Typical standard chromatogram.

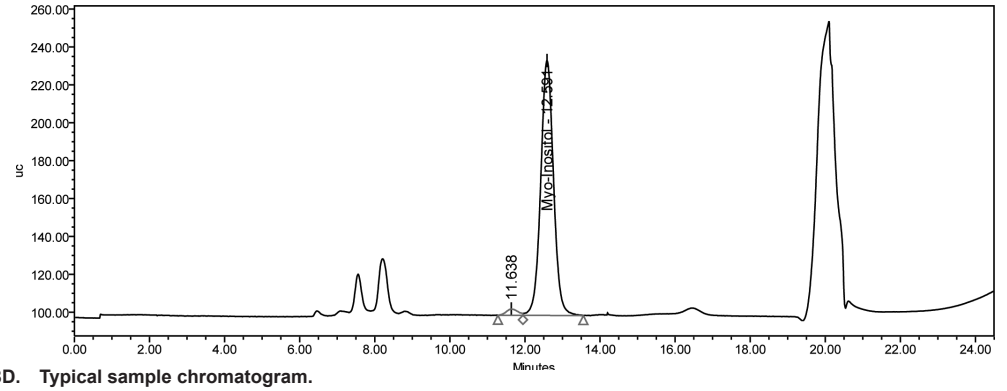

Figure 2011.18D. Typical sample chromatogram.

Table 1. Multilaboratory collaborative study data for free myo-inositol[a]

Lab No.	NIST 1849a		Infant partially hydrolyzed milk powder		Infant partially hydrolyzed soy powder		Child powder		Infant elemental powder		Infant milk powder		Infant soy powder		Infant milk RTF		Infant milk RTF, unfortified	
1	416	415	3.76	3.83	3.33	3.25	5.13	5.11	5.21	5.28	4.26	4.30	4.34	4.30	7.13	7.16	3.23	3.19
2	408	417	2.76[b]	2.76[b]	2.64	2.46	5.62[c]	5.67[c]	5.16	4.99	4.74[c]	4.76[c]	4.04	4.10	7.07	7.12	3.26	3.16
3	404	422	3.60	3.72	3.00	2.81	4.96	4.97	5.16	4.95	4.22	4.21	3.65	4.03	7.09	7.08	3.06	3.05
4	404	421	3.78	3.78	3.23	3.23	5.08	5.10	5.34	5.30	4.41	4.26	4.49	4.27	7.46	7.53	3.10	3.16
5	414	408	3.83	3.85	3.24	3.17	5.14	5.13	4.31[d]	5.33[d]	4.19	4.17	4.40	4.31	NA[e]	NA	NA	NA
6	411	412	3.86	3.90	3.57	3.50	5.22	5.26	5.37	5.32	4.38	4.39	4.60	4.66	7.31	7.34	3.26	3.35
7	406	410	3.90	3.94	3.47	3.50	5.00	5.02	5.53	5.29	4.06	4.11	4.44	4.43	7.40	7.34	3.16	3.19
8	412	420	3.93	3.87	3.27	3.36	4.96	4.92	5.23	5.12	4.24	4.10	4.23	4.32	7.25	7.18	3.16	3.21
	430	435	3.91	3.92	3.48	3.45	4.92	4.91	5.23	5.29	NT[f]	NT	4.41	4.44	7.22	7.10	3.20	3.22
9	378	420	3.80	3.81	2.84	2.80	5.53	4.63	4.96	4.85	4.41[d]	3.71[d]	4.17	3.82	7.11[d]	6.71[d]	2.94	3.13
10	404	403	2.96[b]	2.91[b]	2.54	2.31	4.97[d]	5.03[d]	4.58	4.44	4.24	4.10	3.67	3.66	6.87	6.9	3.14	3.24

[a] Concentration in mg/100 g RTF liquid for all products except NIST 1849a. NIST 1849a concentration in mg/kg powder.

[b] Data excluded. Failed Double Grubbs' test.

[c] Data excluded. Failed Single Grubbs' test.

[d] Data excluded. Failed Cochran's test.

[e] NA = Not applicable. Laboratory did not receive sample.

[f] NT = Not tested. Laboratory did not complete testing.

Table 2. Multilaboratory collaborative study data for phosphatidylinositol bound myo-inositol[a]

Lab No.	NIST 1849a		Infant partially hydrolyzed milk powder		Infant partially hydrolyzed soy powder		Child powder		Infant elemental powder		Infant milk powder		Infant soy powder		Infant milk RTF		Infant milk RTF, unfortified	
1	13.4	13.7	0.296	0.296	2.25	2.11	0.513	0.500	0	0	0.954	0.960	2.93	2.83	0.519	0.522	0.469	0.451
2	11.3	10.0	0.221	0.232	1.91	1.78	0.411	0.405	0	0	0.809	0.724	2.41	2.24	0.361	0.324	0.262	0.278
3	6.25	9.34	0.201	0.188	1.53	1.56	0.322	0.301	0	0	0.583	0.579	2.07	2.20	0.333	0.289	0.290	0.218
4	6.77	8.22	0.160	0.167	1.42	1.36	0.250	0.316	0	0	0.402	0.387	1.34	1.72	0.272	0.341	0.265	0.328
5	8.21[b]	15.3[b]	0.243	0.221	1.93	1.75	0.353	0.351	0	0	0.697	0.754	2.06	2.47	NA[c]	NA	NA	NA
6	NT[d]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
7	10.9	10.9	0.307	0.312	1.41	1.46	0.367	0.340	0	0	0.681	0.654	1.91	1.90	0.448	0.363	0.382	0.352
8	7.50	8.89	0.175	0.166	1.25	1.24	0.198	0.204	0	0	0.454	0.450	1.63	1.56	0.213	0.251	0.199	0.211
	6.15	6.33	0.173	0.189	1.11	1.13	0.233	0.254	0	0	NT	NT	1.40	1.34	0.251	0.260	0.212	0.231
9	7.97	8.48	0.126	0.144	1.85	1.70	0.320	0.274	0	0	0.706	0.682	2.37	2.51	0.360	0.356	0.293	0.295
10	10.2	10.4	0.219	0.243	2.16	1.93	0.331	0.322	0	0	0.757	0.768	2.51	2.53	0.405	0.389	0.381	0.370

[a] Concentration in mg/100 g RTF liquid for all products except NIST 1849a. NIST 1849a concentration in mg/kg powder.

[b] Data excluded. Failed Cochran's test.

[c] NA = Not applicable. Laboratory did not receive sample.

[d] NT = Not tested. Laboratory did not complete testing.

F. Calculations

Before calculating myo-inositol concentrations in samples, compare the myo-inositol standard peaks with the myo-inositol sample peaks and confirm that there are no interfering compounds and that the myo-inositol sample peak areas or heights are within the range of the myo-inositol standard peak areas or heights. The concentration of myo-inositol cannot be calculated if there are interferences or if the separation is poor. The myo-inositol retention time should be 11 to 13 min depending on the individual analytical column. *See* Figures **2011.18C** and **D** for typical standard and sample chromatograms, respectively.

(a) Concentration of working standards:

$$C_W = W \times 1/0.05 \times 1/10 \times A_1/V_1 \times A_2/V_2 \times p = W \times 2 \times A_1/V_1 \times A_2/V_2 \times p$$

where C_W is the concentration of the working standard solution in mg/L; W is the weight, in mg, of myo-inositol standard weighed; 0.05 is the dilution volume of the stock standard in L; 1/10 is the intermediate standard dilution (10 to 100 mL); A_1 is the aliquot of intermediate standard used, in mL; V_1 is the dilution volume of the working standard in mL; A_2 is the aliquot of working standard used, in mL, if applicable; and V_2 is the dilution volume of the working standard in mL, if applicable; and p is the standard purity in mg/mg.

Table 3. Multilaboratory collaborative study data for free plus phosphatidylinositol bound myo-inositol[a]

Lab No.	NIST 1849a		Infant partially hydrolyzed milk powder		Infant partially hydrolyzed soy powder		Child powder		Infant elemental powder		Infant milk powder		Infant soy powder		Infant milk RTF		Infant milk RTF, unfortified	
1	429	429	4.06	4.13	5.57	5.36	5.65	5.61	5.21	5.28	5.21	5.26	7.27	7.13	7.65	7.68	3.70	3.64
2	419	427	2.98[b]	2.99[b]	4.55	4.24	6.03	6.07	5.16	4.99	5.55	5.48	6.46	6.34	7.43	7.44	3.52	3.44
3	411	431	3.80	3.91	4.53	4.37	5.28	5.27	5.16	4.95	4.81	4.79	5.72	6.23	7.42	7.37	3.35	3.27
4	410	429	3.94	3.95	4.65	4.59	5.33	5.42	5.34	5.3	4.81	4.64	5.83	5.99	7.73	7.87	3.36	3.49
5	422	423	4.07	4.07	5.17	4.92	5.5	5.48	4.31[c]	5.33[c]	4.88	4.92	6.47	6.78	NA[d]	NA	NA	NA
6	NT[e]	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
7	417	421	4.21	4.25	4.88	4.95	5.37	5.36	5.53	5.29	4.74	4.77	6.35	6.33	7.85	7.70	3.55	3.55
8	420	429	4.10	4.03	4.52	4.60	5.16	5.13	5.23	5.12	4.7	4.55	5.85	5.89	7.47	7.43	3.36	3.42
	436	442	4.08	4.11	4.59	4.58	5.15	5.16	5.23	5.29	NT	NT	5.81	5.77	7.47	7.36	3.41	3.45
9	386	428	3.93	3.95	4.70	4.49	5.85[c]	4.91[c]	4.96	4.85	5.12[c]	4.39[c]	6.54	6.33	7.47[c]	7.06[c]	3.24	3.43
10	414	413.0	3.18[b]	3.15[b]	4.70	4.24	5.30	5.35	4.58	4.44	5.00	4.87	6.17	6.19	7.27	7.29	3.52	3.61

[a] Concentration in mg/100 g RTF liquid for all products except NIST 1849a. NIST 1849a concentration in mg/kg powder.

[b] Data excluded. Failed Double Grubbs' test.

[c] Data excluded. Failed Cochran's test.

[d] NA = Not applicable. Laboratory did not receive sample.

[e] NT = Not tested. Laboratory did not complete testing.

Table 4. Collaborative study results for free myo-inositol with outliers removed

Sample type	Total No. labs[a]	Total No. replicates[b]	Mean, mg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat
Infant formula (NIST SRM 1849a)	10 (0)	22 (0)	412[c]	11.3	11.4	2.75	2.77	0.43
Infant formula powder soy-based	10 (0)	22 (0)	4.22	0.127	0.305	3.03	7.26	0.80
Infant formula powder milk-based	10 (2)	20 (4)	4.23	0.066	0.109	1.56	2.57	0.28
Infant formula RTF milk-based	9 (1)	20 (2)	7.20	0.046	0.185	0.63	2.57	0.31
Infant formula powder partial hydrolyzed milk-based	10 (2)	22 (4)	3.83	0.037	0.089	0.96	2.33	0.25
Infant formula powder partial hydrolyzed soy-based	10 (0)	22 (0)	3.11	0.090	0.389	2.92	12.6	1.32
Child formula powder	10 (2)	22 (4)	5.05	0.023	0.109	0.46	2.15	0.24
Infant elemental powder	10 (1)	22 (2)	5.13	0.095	0.277	1.85	5.41	0.61
Infant formula RTF milk-based, unfortified	9 (0)	20 (0)	3.17	0.058	0.091	1.84	2.87	0.30

[a] Number of laboratories submitting data (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Data reported as mg/kg powder.

Table 5. Collaborative study results for phosphatidylinositol bound myo-Inositol with outliers removed

Sample type	Total No. labs[a]	Total No. replicates[b]	Mean, mg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat
Infant formula (NIST SRM 1849a)	9 (1)	20 (2)	9.26[c]	1.08	2.37	11.3	25.0	2.19
Infant formula powder soy-based	9 (0)	20 (0)	2.10	0.150	0.501	6.94	23.2	2.30
Infant formula powder milk-based	9 (0)	18 (0)	0.667	0.026	0.172	3.92	25.9	2.15
Infant formula RTF milk-based	8 (0)	18 (0)	0.348	0.030	0.091	8.36	25.2	1.91
Infant formula powder partial hydrolyzed milk-based	9 (0)	20 (0)	0.214	0.010	0.058	4.72	26.4	1.86
Infant formula powder partial hydrolyzed soy-based	9 (0)	20 (0)	1.64	0.094	0.358	5.53	21.1	2.02
Child formula powder	9 (0)	20 (0)	0.328	0.023	0.088	6.89	25.8	1.94
Infant elemental powder	9 (0)	20 (0)	0	0	0	0	0	0
Infant formula RTF milk-based, unfortified	8 (0)	20 (0)	0.305	0.024	0.085	7.71	26.9	2.00

[a] Number of laboratories submitting data (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Data reported as mg/kg powder.

Table 6. Collaborative study results for free plus phosphatidylinositol bound myo-inositol with outliers removed

Sample type	Total No. labs[a]	Total No. replicates[b]	Mean, mg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat
Infant formula (NIST SRM 1849a)	9 (0)	20 (0)	422[c]	11.9	11.9	2.83	2.83	0.44
Infant formula powder soy-based	9 (0)	20 (0)	6.27	0.147	0.446	2.32	7.05	0.82
Infant formula powder milk-based	9 (1)	18 (2)	4.94	0.070	0.302	1.41	6.12	0.69
Infant formula RTF milk-based	8 (1)	18 (2)	7.53	0.059	0.201	0.78	2.66	0.32
Infant formula powder partial hydrolyzed milk-based	9 (2)	20 (4)	4.04	0.038	0.124	0.94	3.07	0.33
Infant formula powder partial hydrolyzed soy-based	9 (0)	20 (0)	4.71	0.152	0.357	3.22	7.55	0.84
Child formula powder	9 (1)	20 (2)	5.42	0.028	0.285	0.51	5.23	0.60
Infant elemental powder	9 (1)	20 (2)	5.11	0.099	0.283	1.94	5.56	0.63
Infant formula RTF milk-based, unfortified	8 (0)	18 (0)	3.46	0.066	0.128	1.90	3.70	0.39

[a] Number of laboratories submitting data (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Data reported as mg/kg powder.

Table 7. Collaborative study results for free myo-inositol with all data included

Sample type	Total No. labs	Total No. replicates	Mean, mg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat
Infant formula (NIST SRM 1849a)	10	22	412[a]	11.3	11.4	2.75	2.77	0.43
Infant formula powder soy-based	10	22	4.22	0.127	0.305	3.03	7.26	0.80
Infant formula powder milk-based	10	20	4.26	0.168	0.232	3.95	5.43	0.60
Infant formula RTF milk-based	9	20	7.17	0.095	0.207	1.33	2.89	0.34
Infant formula powder partial hydrolyzed milk-based	10	22	3.65	0.035	0.412	0.97	11.4	1.22
Infant formula powder partial hydrolyzed soy-based	10	22	3.11	0.090	0.389	2.92	12.6	1.32
Child formula powder	10	22	5.10	0.185	0.246	3.61	4.81	0.54
Infant elemental powder	10	22	5.10	0.227	0.318	4.45	6.24	0.71
Infant formula RTF milk-based, unfortified	9	20	3.17	0.058	0.091	1.84	2.87	0.30

[a] Data reported as mg/kg powder.

Table 8. Collaborative study results for phosphatidylinositol bound myo-inositol with all data included

Sample type	Total No. labs	Total No. replicates	Mean, mg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat
Infant formula (NIST SRM 1849a)	9	20	9.51[a]	1.82	2.62	18.7	26.8	2.36
Infant formula powder soy-based	9	20	2.10	0.150	0.501	6.94	23.2	2.30
Infant formula powder milk-based	9	18	0.667	0.026	0.172	3.92	25.9	2.15
Infant formula RTF milk-based	8	18	0.348	0.030	0.091	8.36	25.2	1.91
Infant formula powder partial hydrolyzed milk-based	9	20	0.214	0.010	0.058	4.72	26.4	1.86
Infant formula powder partial hydrolyzed soy-based	9	20	1.64	0.094	0.358	5.53	21.1	2.02
Child formula powder	9	20	0.328	0.023	0.088	6.89	25.8	1.94
Infant elemental powder	9	20	0	0	0	0	0	0
Infant formula RTF milk-based, unfortified	8	20	0.305	0.024	0.085	7.71	26.9	2.00

[a] Data reported as mg/kg powder.

Table 9. Collaborative study results for free plus phosphatidylinositol bound myo-inositol with all data included

Sample type	Total No. labs	Total No. replicates	Mean, mg/100 g RTF	SD_r	SD_R	RSD_r, %	RSD_R, %	HorRat
Infant formula (NIST SRM 1849a)	9	20	422[a]	11.9	11.9	2.83	2.83	0.44
Infant formula powder soy-based	9	20	6.27	0.147	0.446	2.32	7.05	0.82
Infant formula powder milk-based	9	18	4.92	0.184	0.314	3.74	6.38	0.72
Infant formula RTF milk-based	8	18	7.50	0.106	0.218	1.41	2.90	0.35
Infant formula powder partial hydrolyzed milk-based	9	20	3.84	0.035	0.426	0.91	11.2	1.21
Infant formula powder partial hydrolyzed soy-based	9	20	4.71	0.152	0.357	3.22	7.55	0.84
Child formula powder	9	20	5.42	0.203	0.307	3.73	5.63	0.64
Infant elemental powder	9	20	5.08	0.237	0.324	4.67	6.40	0.72
Infant formula RTF milk-based, unfortified	8	18	3.46	0.066	0.128	1.90	3.70	0.39

[a] Data reported as mg/kg powder.

(b) *Preparation of standard curve.*—For each working standard concentration, average the peak areas or heights from each of two consecutive sets of standards. Prepare a standard curve by performing linear least squares regression on the concentrations versus the averaged peak areas or heights. A standard curve must have a correlation of at least 0.999 to be considered acceptable for sample calculations.

At each working standard level, the peak areas or heights of standards injected before and after a set of samples must not increase or decrease by more than 7%.

(c) *Calculation of myo-inositol in samples.*—The concentration of myo-inositol in a prepared sample is extrapolated from the standard curve prepared above. From the diluted, prepared sample concentration, the product concentration can be calculated:

$$C_p = (C_d \times D_1)/S$$

where C_p is the concentration of myo-inositol in the product sample in mg/kg, C_d is the concentration of myo-inositol in the prepared sample in mg/L, D_1 is the dilution volume in mL, and S is the sample weight in g.

Note: For each set of samples, the control result must be within 3 SDs of the control mean.

Results and Discussion

All of the free, phosphatidylinositol bound, and free plus phosphatidylinositol bound myo-inositol collaborative study data are summarized in Tables 1–9. It should be noted that laboratory 5 did not receive the liquid RTF study samples and that laboratory 6 was not able to complete the phosphatidylinositol bound myo-inositol testing.

Using the AOAC INTERNATIONAL Interlaboratory Study Blind (Unpaired) Replicates Workbook (7), statistical outliers from one or two laboratories were identified in five of the nine products analyzed for free plus phosphatidylinositol bound myo-inositol content. After removal of outliers, RSD_r ranged from 0.51 to 3.22% and met the SMPR of ≤5% for all the products analyzed. The RSD_R ranged from 2.83 to 7.55% and met the SMPR of ≤8% for all the products analyzed. When the outliers were included, RSD_r ranged from 0.91 to 4.67%, meeting the SMPR of ≤5% for all the products analyzed, and the RSD_R ranged from 2.83 to 11.2%, meeting the SMPR of ≤8% for eight of the nine products analyzed.

Since it is possible that some laboratories may only use this method for free myo-inositol analyses, a review of the free myo-inositol collaborative data is also included here. Using the AOAC INTERNATIONAL Interlaboratory Study Blind (Unpaired) Replicates Workbook, statistical outliers from one or two laboratories were identified in five of the nine products analyzed for free myo-inositol content. After removal of free myo-inositol outliers, RSD_r ranged from 0.46 to 3.03% and met the SMPR of ≤5% for all the products analyzed. The RSD_R ranged from 2.15 to 12.6% and met the SMPR of ≤8% for eight of the nine products analyzed. When the outliers were included in the free myo-inositol data summary, RSD_r ranged from 0.97 to 4.45%, still meeting the SMPR of ≤5% for all the products analyzed, and the RSD_R ranged from 2.77 to 12.6% and met the SMPR of ≤8% for seven of the nine products analyzed.

Several laboratories provided comments about the method. Some laboratories made positive comments regarding the column switching format because it saved time and kept the electrode clean, while other laboratories would prefer using a gradient rather than column switching. One laboratory questioned the need for determining the phosphatidylinositol component. As noted previously, some laboratories had to use a higher temperature than that listed in the method to improve recoveries of phosphatidylinositol bound myo-inositol, and some laboratories had trouble adding 50% sodium hydroxide directly to the samples immediately after hydrolysis. One laboratory recommended adding additional guidance for determining the amount of acid needed to adjust the pH of a sample to 4.5 for free myo-inositol analyses and for determining the percentage recovery of phosphatidylinositol from the SPE cartridges. One laboratory noted that the method should specify that the hydrolysis procedure be performed in a fume hood. Additional information was added to AOAC Final Action Method **2011.18** to address some of study participants' comments and concerns listed above.

Conclusions

AOAC Method **2011.18** was collaboratively studied by nine to 10 laboratories from five different countries with a variety of infant and pediatric nutritional matrixes. The method demonstrated acceptable repeatability and reproducibility and met the SPIFAN SMPRs for free and free plus phosphatidylinositol bound myo-inositol in most of the matrixes analyzed.

Recommendation

The multilaboratory collaborative study data were summarized and presented to the AOAC ERP in September 2014. After reviewing the data, the ERP voted to move AOAC **2011.18** to Final Action status, and the method was approved by the AOAC Official Methods Board as a Final Action Method.

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Chromium, Selenium, and Molybdenum in Infant Formula and Adult Nutritional Products by Inductively Coupled Plasma/Mass Spectrometry: Collaborative Study, Final Action 2011.19

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AOAC First Action Method 2011.19: Chromium, Selenium, and Molybdenum in Infant Formula and Adult Nutritional Products, was collaboratively studied. This method uses microwave digestion of samples with nitric acid, hydrogen peroxide, and internal standard followed by simultaneous detection of the elements by an inductively coupled plasma (ICP)/MS instrument equipped with a collision/reaction cell. During this collaborative study, nine laboratories from four different countries, using seven different models of ICP/MS instruments, analyzed blind duplicates of seven infant, pediatric, and adult nutritional formulas. One laboratory's set of data was rejected in its entirety. The method demonstrated acceptable repeatability and reproducibility and met the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) *Standard Method Performance Requirements* (SMPRs®) for almost all of the matrixes analyzed. The Cr, Mo, and Se SPIFAN requirement for repeatability was ≤5% RSD. The SMPR called for a reproducibility of ≤15% RSD for products with ultratrace element concentrations above the targeted LOQ of 20 µg/kg Cr/Mo and 10 µg/kg Se (as ready-to-feed). During this collaborative study, RSD_r ranged from 1.0 to 7.0% and RSD_R ranged from 2.5 to 13.4% across all three ultratrace elements.

Most infant formulas are fortified with the essential trace element selenium (Se); many pediatric and adult nutritional products are also fortified with chromium (Cr) and molybdenum (Mo; 1, 2). Together these ultratrace elements represent some of the most difficult analyses for any laboratory testing against the relatively narrow specification ranges mandated by many regulatory bodies for these added nutrients in infant and pediatric formulas. Existing official methods that have been specifically validated for Cr, Mo, and Se in infant and pediatric nutritional products were virtually nonexistent until the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) issued a Call for Methods in 2011 and a suitable method was put forward that was already being used in the authors' laboratory (3). This method appeared to have the requisite precision, accuracy, and ruggedness for ultratrace element analysis based upon successful completion of an internal single-laboratory validation (SLV). The SPIFAN Working Group had formulated a set of *Standard Method Performance Requirements* (SMPRs®) that captured the needs of the formula manufacturers for a suitable global dispute resolution method for ultratrace element analysis. SMPRs were approved by stakeholders (4), and the AOAC Expert Review Panel (ERP) on SPIFAN Nutrient Methods approved only this method as First Action in 2011 (AOAC **2011.19**) because it appeared to meet the SMPRs on that manufacturer's own products. However, the ERP requested the SLV be repeated with the specific set of SPIFAN matrixes developed to be representative of most of the other manufacturers' products. After examining the second set of SLV data, the ERP voted that the method proceed to a multilaboratory testing (MLT) of reproducibility. These latter SLV data have not been published before and are captured in this report with the subsequent MLT collaborative data using AOAC method **2011.19**.

Multilaboratory Collaborative Study

Initially 16 laboratories expressed interest in participating in the AOAC **2011.19** ultratrace element collaborative study, but only nine laboratories were able to complete the study because of lack of time or resources, or they could not import the samples in due time. The nine participating laboratories were located in four different countries (China, United States, India, and France) and were equipped with seven different models of inductively coupled plasma (ICP)/MS instruments, namely the PerkinElmer (Shelton, CT) ELAN DRC-e, ELAN DRC II, and NexION 300D; the Agilent (Santa Clara, CA) 7700x and 7500cx (the latter used only during the authors' SLV); and the Thermo Scientific (Waltham, MA) iCAP Q and X Series 2. All these instruments

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The method was approved by the AOAC *Official Methods Board* as Final Action. *See* "Standards News," (2014) *Inside Laboratory Management*, November/December 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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were equipped with modern collision/reaction cells that are thought to be necessary to avoid the Ar/C spectral interferences on the major Cr and Se isotopes.

Before actual MLT study samples were analyzed, each participating laboratory was asked to set up the method and evaluate the linearity and the method LOQ with their given instrument model. This exercise is identical to what is done to transfer a mineral method to another site in the authors' internal laboratory network, as it quickly identifies problems in procuring or preparing suitable standards and standard blanks, or in otherwise setting up the instrument parameters. To check the linearity, standards were analyzed and the calibration curve prepared on each of 3 separate days. On each day, working standards at the lowest concentration level (WS1) and at ½ WS1 were analyzed as samples, and then their calculated concentrations were compared to their nominal concentrations. The mean recovery of each standard versus its nominal concentration (i.e., the calibration residual) had to be within 5%. All laboratories passed this test except Laboratories 9 and 11, both of which failed only at the lowest standard level for Se (Table 1). For these laboratories, the practical LOQ (PLOQ) for Se was therefore equal to WS1, whereas the other laboratories could analyze as low as ½ WS1 in concentration.

The second setup test was to analyze the sample blank on 5 separate days (done at same time as the linearity study, plus two more days), calculating the SD of these results, multiplying that by 10, and then adding that result to the blank mean. This LOQ was multiplied by the method's dilution factor of 50 to arrive at the approximate LOQ in terms of sample weight. The SMPRs state an LOQ of 20 ng/g Cr and Mo and 10 ng/g Se on a ready-to-feed (RTF) basis. Table 1 shows the prework results from the participating laboratories (Laboratories 6 and 7 dropped out about this time). Note that it is desirable to have low, consistent blanks for good sensitivity, as well as the linearity, to avoid excessive calibration bias. These trials immediately pointed to Laboratories 1 and 10 as having potential problems; they were allowed to proceed with the MLT, but indeed Laboratory 1's data were eventually rejected in total. The prework results for Laboratory 10 may not have been so ominous because it did not submit all the data, and the PLOQs could not be calculated.

The final prework for the participating laboratories was to analyze the NIST Standard Reference Material (SRM) 1849a sample. All laboratories passed this test by producing Cr, Mo, and Se results within 5% of the certified means (data not shown, but similar to data collected during the MLT, which is tabulated later). The fact that Laboratories 1 and 10 produced good results on the SRM might be attributed to the relatively high concentration of these elements in the SRM. It should be noted that six of the nine laboratories determined Na, K, P, Mg, Ca, Fe, Cu, Zn, and Mn concurrently with the Cr, Mo, and Se with good precision and accuracy. This work was done under the direction of the Study Director. Results on SPIFAN samples are published in this issue of *J. AOAC Int.* [AOAC First Action Method **2015.06** by Thompson, J.J., Pacquette, L., & Brunelle, S.L. (2015) *J. AOAC Int.* **98**, 1711–1720]. Method **2011.19** appears to be viable as a 12-element method, not just as a method for ultratrace elements.

Each participating laboratory received blind duplicates of seven of the SPIFAN matrixes (this study used the original SPIFAN set) for a total of 14 samples to test. NIST SRM 1849a was not included as a blind sample, but rather the participants were instructed to analyze it concurrently with the other samples as if it were a control sample. The seven matrixes tested were an infant formula partially hydrolyzed milk-based powder, an adult nutritional low-fat powder, an adult nutritional milk protein-based powder, a child formula powder, an infant elemental powder, an adult high protein nutritional RTF liquid, and an adult high-fat nutritional RTF liquid. Only two infant formula types were chosen (there were four more in the SPIFAN set) because they were known to be unfortified in Cr and Mo and would not yield useful information.

Participants were asked to reconstitute all powders prior to analysis with the exception of SRM 1849a, which was unblinded but rather easily identified by its sachet anyway. Participants used a direct weight of 0.2 g SRM powder, which has proven to be homogeneous for minerals at this weight through extensive use in the authors' laboratories. All other powders were reconstituted by either dissolving 20 g powder in enough laboratory water to make 200 g solution, i.e., a 10% (w/w) reconstitution, or by following the official method with the SPIFAN-recommended 25 g sample + 200 g water (11.1%, w/w). Some laboratories asked to work with the 10% reconstitution rates, as this is certainly an easier

Table 1. Set-up tests for participating laboratories[a]

Lab	LOQ Cr (20 ng/g required), ng/g	PLOQ Cr, µg/L	LOQ Mo (20 ng/g required), ng/g	PLOQ Mo, µg/L	LOQ Se (10 ng/g required), ng/g	PLOQ Se, µg/L
1	45	0.4	30	0.4	46	0.2
2	7	0.4	5	0.4	4	0.2
3	9	0.4	9	0.4	3	0.2
4	12	0.4	2	0.4	1	0.2
5	9	0.4	1	0.4	6	0.2
6						
7						
8	4	0.4	1	0.4	1	0.2
9	16	0.4	13	0.4	18	0.4
10	44	?	14	?	66	?
11	8	0.4	4	0.4	13	0.4

[a] Laboratories 6 and 7 dropped out at this time and Laboratory 10's data were incomplete. A PLOQ of 0.4 µg/L Cr/Mo and 0.2 µg/L Se along with an LOQ below 20 ng/g (10 ng/g for Se), was desired. *See* text for details.

factor with which to work. The Study Director allowed this small variation to the method as it will certainly not affect the validity of results if the correct dilution factor is used.

Participants were reminded more than once that the written (now official) method mandates the use of several QC/system suitability solutions including a blank check (must be less than the PLOQ), a calibration verification standard (must be within 4% of its nominal concentration before and after samples are run), and a control sample (the concurrent analysis of SRM 1849a). The criterion for results was not explicitly stated because these laboratories do not have working control charts for such; however, the SRM expected results were known from the prework phase, and the laboratories could see the SRM results concurrent with their samples. No laboratories indicated they discarded any data because of these QC solutions failing.

A final key suitability requirement was the analysis of duplicates that had to agree within 10% for Cr, 7% for Se, and 5% for Mo. These duplicates are not to be confused with the blinded duplicates supplied in the collaborative study. The duplicate requirement is present not so much to improve the confidence interval by using the mean of two results (although that can be useful for concentrations near the PLOQ), but rather to indicate the presence of substantial indeterminate errors before the data are allowed to enter the pool of multilaboratory data. A 10% agreement between duplicates is a common, if somewhat arbitrary, criterion used in many GB (China) official methods, and others. In this case, the SLV and MLT data indicate that a 10% criterion is well above the excellent repeatability or intermediate precision expected of this method (*see* SLV data later in this report), but this level is maintained for Cr because small levels of Cr contamination were sometimes unavoidable and usually irrelevant to results since most adult products had relatively high levels of Cr (infant formulas are not fortified with Cr or Mo). With the inclusion of the other QC tests in this method, especially the use of the control sample, the possibilities of other sources of indeterminate errors are small [e.g., pipetting the wrong amount of internal standard (IS) or a poorly made set of standards], and so Se and Mo have duplicate RSD requirements closer to 3× the typical short term precision of about 1.5–2.0%. Indeed, the expected duplicate precision for Se was originally set at 5% RSD for this method, which was optimal for the authors' laboratories, but analysis of these MLT data indicated too many rejections at that level, and so a 7% RSD requirement is now set for Se. The requirement for Mo is still 5% duplicate precision. Table 2 shows the number of failures in analyzing the 14 MLT samples using the original criteria (i.e., 5% RSD duplicate precision for Se). There are an inordinate number of failures (4/14 or almost 30%) for Laboratory 1 Cr results; this is the laboratory whose data were later entirely rejected from the study. However, it appears that only Laboratory 1 had this problem with Cr determination. In contrast, five laboratories had more than one sample rejected for Se when the duplicate RSD criterion were set to 5%. If the duplicate precision criterion was set to 7% RSD, only eight total failures occurred instead of the 19 shown in Table 2, out of a total of nine laboratories × 15 samples = 135 determinations, or about a 6% rejection rate. This may be higher than the <1% rejection rate for Mo because Se concentrations are routinely low, about 2–3x above the PLOQ in all the samples tested. Also, the laboratory that had the most Se data rejected, Laboratory 9, was also the one that had a compromised PLOQ as shown in Table 1. This underscores the importance of having optimal sensitivity for Se analyses in infant/pediatric formulas. Generally, we have observed that ICP/MS units that are not fitted with hydrogen gas for collisional reaction/reduction of argon interference cannot readily obtain the 0.2 ppb PLOQ in solution. As can be seen, setting a single criterion for duplicate precision to cover all concentration levels encountered and for all matrixes is difficult, but this does not mean it should not be done. This is perhaps the best way to avoid out-of-specification results due to systematic errors and rejecting the data before any unnecessary retesting or regulatory action begins, and this kind of suitability criterion should be strongly considered for any dispute resolution method, even chromatography-based methods in which it may take much more time to get the duplicate result.

Table 2. MLT duplicate samples failing to meet the original duplicate precision criterion of 10% RSD for Cr and 5% RSD for Se and Mo

Lab	No. Cr failures	No. Mo failures	No. Se failures[a]
1	4	0	1 (0)
2	0	0	0
3	0	0	3 (1)
4	1	0	2 (0)
5	1	1	4 (2)
6			
7			
8	0	0	0
9	0	0	5 (4)
10	1	0	4 (1)
11	0	0	0

[a] Failures under 7% RSD criterion shown for Se in parenthesis.

Upon completion of the sample analyses, participating laboratories were asked to send all of their data to the Study Director. An Excel spreadsheet was supplied by the Director, with a template for adding the sample weights, duplicate results, and spaces for all the calibration and QC results. Participants were also asked to report any deviations to the method and any relevant comments based on their experiences with the method.

All data were statistically analyzed in a spreadsheet (5) using AOAC INTERNATIONAL guidelines to determine overall mean, repeatability SD (s_r), RSD_r, reproducibility SD (s_R), RSD_R, and Horwitz ratio (HorRat). Cochran's (P = 0.025, one-tail) and Grubbs' (single and double, P = 0.025, two-tail) tests were used to determine statistical outliers.

SPIFAN SMPRs for repeatability were ≤5% RSD and requirements for reproducibility were ≤15% RSD in products above a concentration of 10 ng/g Se and 20 ng/g Cr/Mo on an RTF basis.

Method

The Final Action method, as now published (6) and given below, is the updated version the participants used for this study. In particular, Ge was substituted as the IS for Ni, Cr, and Mo, and there is an option to analyze more elements concurrently. The QC/system suitability was more explicitly stated, and the revised 7% duplicate criterion for Se added.

AOAC Official Method 2011.19
Chromium, Selenium, and Molybdenum in Infant Formula and Adult Nutritional Products
Inductively Coupled Plasma (ICP/MS)/Mass Spectrometry
First Action 2011
Final Action 2014

ISO-IDF–AOAC Method

A. Principle

A test portion is heated with nitric acid in a closed vessel microwave digestion system at 200°C. Digested test solution, or an appropriate dilution, is analyzed with the ICP/MS instrument standardized with acid matched standard calibrant solutions. An ionization buffer (K) is used to minimize easily ionizable element (EIE) effects, methanol is added to normalize the C content, and Ge and Te are used as ISs. It is permissible to combine the analysis of Cr/Mo/Se with simultaneous determination of any or all of these elements: Na, K, P, Mg, Ca, Fe, Zn, Cu, and Mn.

B. Apparatus

(a) *Microwave oven system.*—Commercial microwave oven system designed for laboratory use at 0–300°C, with closed vessel system and controlled temperature ramping capability. It is recommended that a vessel design be selected that will withstand the maximum possible pressure, since organic material, and carbonates if not given sufficient time to predigest, will generate significant pressure during digestion. Vent according to manufacturer's recommendation. (*Caution*: Microwave operation involves hot pressurized acid solution. Use appropriate face protection and laboratory clothing.) Additional instrument parameters are summarized in Table **2011.19A**.

(b) *ICP mass spectrometer.*—With collision reaction cells. In the multilaboratory testing study, five different ICP/MS instrument models from three major vendors delivered equivalent performance.

Table 2011.19A. ICP/MS parameters

Typical operating conditions	
RF power, W	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)
Analyte/IS/gas mode	^{52}Cr, $^{95}Mo/^{72}Ge$ in He mode $^{78}Se/^{130}Te$ in H_2 mode

(c) *Various plasticware and pipets.*

C. Reagents

[*Caution*: Use normal laboratory safety precautions (laboratory coats and safety glasses with side shields) when handling concentrated acids, bases, and organic solvents. Additional protections such as face shields, neoprene gloves, and aprons should be used where splashing may occur. Avoid breathing vapors by working in approved hoods.]

(a) *Laboratory water.*—Use 18 MΩ water throughout for dilution.

(b) *Concentrated nitric acid (HNO_3).*—65–70% trace metal-grade HNO_3 (J.T. Baker, Phillipsburg, NJ) or equivalent throughout.

(c) *Hydrogen peroxide.*—30% ACS reagent grade (J.T. Baker) or equivalent.

(d) *Methanol.*—99.99% analytical reagent grade (EMD Chemicals Inc., Gibbstown, NJ) or equivalent for matrix matching.

(e) *Potassium stock solution.*—10 000 mg/L K (High Purity Standards, Charleston, SC) or equivalent.

D. Standards

(a) *2 mg/L Cr and Mo and 1 mg/L Se multielement stock standard solution in HNO_3.*—High-Purity Standards or equivalent.

(b) *5 mg/L Ge and Te multielement stock standard solution in HNO_3.*—High-Purity Standards or equivalent.

(c) *SRM 1849a.*—National Institute of Standards and Technology (NIST; Gaithersburg, MD) or other suitable SRM to serve as a control for this analysis.

E. Procedure

(a) *Standard preparation.*—Prepare intermediate standards from commercial stock standards at 40 ng/mL Cr and Mo and 20 ng/mL Se. Custom-blended multielement stock standard in HNO_3 is acceptable. Prepare three multielement working standards containing 0.8, 4.0, and 20 ng/mL Cr and Mo and 0.4, 2.0, and 10 ng/mL Se, plus blank, with both 50 ng/mL Ge and Te ISs in HNO_3. Ge is used as the IS for both Cr and Mo, and Te must be used for Se.

(b) *Sample preparation.*—Prepare powder samples by reconstituting approximately 25 g sample in 200 mL warm laboratory water (60°C). Accurately weigh approximately 1.8 g reconstituted test portion into the digestion vessel. This represents approximately 0.2 g original powder sample. SRM 1849a is weighed at 0.2 g directly into microwave vessel. Fluid samples may be prepared by accurately weighing approximately 1 g test portion weighed directly into the digestion vessel after mixing. For the recommended one-step digestion (two stages in microwave program), add 0.500 mL 5000 ng/mL Ge and Te IS solution (with a micropipette calibrated at point-of-use to deliver the target volume with a tolerance of ±0.8%: do not add the ISs online) and 5 mL trace metal-grade HNO_3 followed by 2 mL H_2O_2 to the microwave digestion vessels. Seal vessels according to manufacturer's directions and place in microwave. Ramp temperature from ambient to 180°C in 20 min, and hold for 20 min in stage 1. In stage 2, the microwave

Table 2011.19B. Microwave operating parameters

	Stage 1 sample digestion	
1	100% power, W	1600
2	Ramp to temperature, min	20
3	Hold time, min	20
4	Temperature, °C	180
5	Cool down, min	20
	Stage 2 sample digestion	
1	100% power, W	1600
2	Ramp to temperature, min	20
3	Hold time, min	20
4	Temperature, °C	200
5	Cool down, min	20

will automatically ramp to 200°C in 20 min and hold for 20 min (*see* Table **2011.19B**).

For microwave ovens without the 2-stage program and where it is more convenient, use the 2-step digestion. Add 0.500 mL 5000 ng/mL Ge and Te IS solution (with a calibrated micropipette at point-of-use) and 5 mL trace metal-grade HNO_3. Do not add the ISs online. With power settings appropriate to the microwave model and number of vessels, ramp temperature from ambient to 200°C in 20 min. Hold at 200°C for 20 min. Cool vessels according to manufacturer's directions, approximately 20 min. Slowly open the microwave vessels, venting the brownish nitrogen dioxide gases. (*Caution:* Venting must be performed in a hood because NO_2 is very toxic.) Add 1 mL H_2O_2 and redigest samples by ramping the temperature from ambient to 180°C in 15 min. Hold at 180°C for 15 min and cool for 20 min.

(c) *Preparation of test solution*.—Add approximately 20 mL laboratory water to the contents of the vessel with the digested samples and transfer to a 50 mL sample vial. Rinse the vessel and transfer the rinsate into the sample vial. Add 0.5 mL methanol to the sample vial and dilute to 50 mL with laboratory water (alternatively, the methanol may be added online at 1%, v/v).

F. Determination

Table **2011.19A** summarizes typical instrument parameters for analysis. Analyze test solutions using an ICP/MS instrument standardized with the indicated standard solutions. Ge is used as the IS for both Cr and Mo (He mode), and Te must be used for Se (H_2 mode). Analyze a 4 ng/mL Cr and Mo and a 2 ng/mL Se working standard 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 method blank (run as a sample; its measured concentration must be <½ of the lowest calibration standard), a duplicate sample [relative percentage difference (RPD) ≤ within 10% for Cr, 7% for Se, and 5% for Mo], and known reference materials serving as control samples (recovery check within control limits) are mandatory for good method performance. If any of these QC checks fails, results should be considered invalid. The order of analysis should be calibration standards, followed by rinse, blank check, check standard, control sample, sample, sample duplicate (up to 10 samples), and finally check standard.

G. Calculations

Sample concentrations were automatically calculated by the software using a nonweighted least-squares linear regression calibration analysis to produce a best-fit line:

$$Y = ax + \text{blank}$$

The analyte concentration in the sample was then calculated:

$$x = \frac{y - \text{blank}}{a} \times \text{DF}$$

where x = analyte concentration (ng/g); y = sample response ratio (ng/mL), which is the measured count of each analyte's standard solution data point in the calibration curve divided by the ratio of the counts/concentration of the IS at the same level; blank = blank standard solution (ng/mL), which is the measured count of the blank standard solution data point in the calibration curve divided by the ratio of the counts/concentration of the IS at the same level as the blank standard solution; a = slope of the calibration curve; and DF = dilution factor of the sample solution (mL/g).

H. Method Validation

(a) *Linearity*.—All calibration curves were prepared using nonweighted least-squares linear regression, and correlation coefficient (r) values were calculated with each calibration curve. Each calibration curve was prepared with four multielement standard solutions, including the blank standard solution. It should be noted that all analyte concentrations in samples were within linear range of the calibration curve and above the established lower linearity limit.

(b) *LOQ*.—The LOQ is the lowest concentration of the analyte in the sample that can be reliably quantitated by the instrument. The method LOQ is typically determined by multiplying the average SD of 10 digested blanks by a factor of 10, and the instrument LOQ by multiplying the instrument LOD by 3 (1). However, in this method the useful LOQ, or practical LOQ (PLOQ), was determined to be the lower linearity limit value of the calibration curve because the accuracy and precision of sample measurements below that value would be uncertain. Almost all mineral-fortified nutritional products can be prepared with a DF such that Cr, Se, and Mo will be present in the analytical solution above the PLOQ.

(c) *Matrix matching with methanol*.—The presence of C (organic compounds) in analytical solutions causes signal enhancement of Se during analysis by ICP/MS (2–4). To determine the optimum concentration of methanol (source of C) needed to compensate for Se signal enhancement, various concentrations of methanol were added to both calibration standards and digested samples.

(d) *Effects of EIEs*.—Many nutritional products contain significant levels of EIEs, such as Ca, Na, K, and Mg. Therefore, blank solutions and solutions containing 4 ng/mL Cr and Mo and 2 ng/mL Se were analyzed both with and without EIEs to determine any changes in concentrations of the analytes.

(e) *Specificity*.—Specificity of the method is its ability to accurately measure the analyte in the presence of other components in the sample matrix that might cause spectral interferences. To demonstrate the specificity of the method, undigested blank solutions were spiked with multielement

Table 3. SLV intermediate precision data for Cr, Mo, and Se (from 8 independent days of testing in duplicate, 4 days per each analyst using two different Agilent ICP/MS units)[a]

Sample type	Cr Mean	RSD_{IP}	Mo Mean	RSD_{IP}	Se Mean	RSD_{IP}
Infant powder milk	4.9 (<PLOQ)	7.7	17.7 (<PLOQ)	8.2	25.6 (<PLOQ)	6.5
Infant powder soy	8.1 (<PLOQ)	5.2	36.5 (<PLOQ)	4.5	24.8 (<PLOQ)	3.3
Infant powdered milk partially hydrolyzed	2.4 (<PLOQ)	20	20.4 (<PLOQ)	4.0	26.6 (<PLOQ)	2.7
Infant powdered soy partially hydrolyzed	6.0 (<PLOQ)	9.2	33.0 (<PLOQ)	3.6	26.8 (<PLOQ)	4.1
Adult powder low fat	47.6	1.3	62.7	3.0	29.7	3.2
Adult powdered milk	15.8 (<PLOQ)	5.0	32.6 (<PLOQ)	4.9	24.3 (<PLOQ)	2.8
Child formula powdered	30.2	1.3	30.0	5.1	23.7	2.9
Infant elemental powdered	23.9	6.3	17.8	7.5	23.2	2.8
Infant RTF milk	7.6 (<PLOQ)	53	16.9 (<PLOQ)	10	29.6 (<PLOQ)	2.9
Adult RTF high protein	130	2.9	154	3.2	92.6	3.0
Adult RTF high fat	141	1.8	193	2.6	133	3.6

[a] Units of concentration are µg/kg as RTF with powders reconstituted 11.1% (w/w). Note that several of the means are designated as <PLOQ and their RSDs are correspondingly higher. PLOQ = practical limit of quantitation.

solutions at concentrations that are representative of nutritional products in samples for ICP/MS analysis. The typical H_2 gas mode for Se, and He gas mode for Cr and Mo, were used.

(f) *Accuracy*.—Accuracy was demonstrated by analyzing three NIST SRMs on 2 independent days, measuring spike recoveries in 10 nutritional products on 3 different days, and comparing results for 10 nutritional products obtained by this method to results obtained by other in-house validated ICP-atomic emission spectrometry and atomic fluorescence spectrometry methods. The spike levels of the analytes added to the products were between 50 and 200% of the analyte concentrations in each product.

(g) *Precision*.—Both within-day and between-day RSD values were determined by analyzing two in-house laboratory control samples. Within-day precision was determined by analyzing the laboratory control samples in duplicate on each day, and between-day precision was measured by using the mean results of the duplicate samples analyzed on each day on 10 different days.

(h) *Ruggedness and robustness*.—To determine the ruggedness of the method, laboratory control samples were analyzed by two analysts on 10 different days. Also, NIST SRM 1849 was analyzed in triplicate with varying sample weights and with different ISs.

(i) *Reproducibility*.—Eight laboratories completed a multilaboratory testing protocol with this method on seven samples submitted as blind duplicates (14 total samples analyzed plus the SRM 1849a control, which was not blinded). Represented were four countries and five ICP/MS instrument models from three major vendors. Results showed an average RSD_R of 9.3% for Cr, 5.3% for Mo, and 6.5% for Se, with an average HorRat ratio of 0.35 across all three analytes and samples.

Table 4. SLV overspike recoveries (mean from 3 days, in triplicate each day) from SPIFAN matrixes at 50–200% of native levels[a]

Product	Cr	RSD	Mo	RSD	Se	RSD
Infant powder milk	109	10.0	92.5	2.5	106	4.7
Infant powder soy	108	1.5	93.1	1.2	103	6.0
Infant powdered milk partially hydrolyzed	90.1	1.1	95.2	5.7	99.5	2.3
Infant powdered soy partially hydrolyzed	91.0	3.0	108	1.0	101	2.3
Adult powder low fat	101	4.8	95.4	8.1	99.3	3.3
Adult powdered milk	104	1.6	97.4	4.6	103	4.1
Child formula powdered	102	2.5	96.2	2.1	104	4.8
Infant elemental powdered	102	8.8	96.9	2.7	106	4.6
Infant RTF milk	103	6.1	92.3	5.4	101	4.9
Adult RTF high protein	98.0	2.5	92.0	2.7	104	4.9
Adult RTF high fat	98.0	2.8	93.3	3.1	105	7.0

[a] Native levels that are below the PLOQ and other concentrations can be found in Table 3.

SLV Data

The SLV data were not published with the method above; they will be briefly summarized here. Table 3 shows the intermediate precision for each of the SPIFAN materials across 8 different days of results using two different analysts and two Agilent ICP/MS units, a model 7500 and a model 7700. As noted previously, the infant formulas contained no added Cr or Mo, so they were mostly not included in the MLT. For levels above the PLOQ,

Table 5. SRM 1849a results during SLV (n = 16, two analysts, two Agilent instruments) and collaborative study (run once in duplicate as a known sample by each laboratory, together with other blinded samples)

	Cr	Mo	Se
Mean SLV, µg/kg, n = 16	105	166	82.7
RSD SLV, %	1.4	1.1	1.8
Certified interval, µg/kg	104.0–110.4	166.7–174.7	78.3–84.1
Mean MLT (not including SLV), µg/kg	106	167	82.6
RSD MLT, % (eight labs)	2.4	2.8	1.5

Table 6. RSD_r, RSD_R, and HorRat values for 2011.19 collaborative study[a]

Matrix	Cr, µg/kg RTF	Labs	RSD_r, %	RSD_R, %	HorRat	Mo, µg/kg RTF	Labs	RSD_r, %	RSD_R, %	HorRat	Se, µg/kg RTF	Labs	RSD_r, %	RSD_R, %	HorRat
Infant powdered milk partially hydrolyzed	<PLOQ	8	N/A	N/A	N/A	20	8	3.3	6.7	0.33	27	8	2.4	2.5	0.13
Adult powder low fat	48	8	4.7	7.1	0.39	63	8	1.6	3.1	0.18	30	8	5.9	7.2	0.37
Adult powdered milk	16	7	3.4	12.1	0.57	33	7	1.0	7.9	0.42	24	8	6.1	6.1	0.31
Child formula powdered	30	7	5.5	9.2	0.48	30	8	3.3	4.6	0.24	24	8	3.8	7.3	0.37
Infant elemental powdered	24	7	3.8	13.4	0.67	18	8	1.7	7.9	0.38	23	8	6.4	9.3	0.46
Adult RTF high protein	130	8	7.0	8.1	0.37	150	7	1.0	3.0	0.14	93	8	2.3	8.1	0.36
Adult RTF high fat	140	8	2.1	5.8	0.27	190	8	1.2	3.8	0.19	133	8	4.7	5.0	0.23
Average			4.4	9.3	0.46			1.9	5.3	0.27			4.5	6.5	0.32

[a] RSDs are shown for means that are at or slightly below PLOQ of 20 µg/kg RTF for Cr and Mo because they are indicative of method performance. No results are above the SMPR required 15% RSD_R. For the five cases in which the number of participating laboratories is listed as $n = 7$, the eighth laboratory's data could be included and still meet SMPR reproducibility requirements (*see* footnotes for Tables 7–9).

intermediate precision was mostly in the range of 2–5% RSD, with the highest at 6.5% RSD. These results are consistent with the subsequent collaborative study, for which reproducibility was about 2–3% higher than the intermediate precision. Repeatability was not probed extensively in the SLV because the short-term precision is very good and the method, after all, does require the duplicate precision to be below the 5% RSD required by the SMPR.

Accuracy was checked via overspike recoveries in the SLV. Table 4 shows the mean recovery of triplicate overspikes on each of 3 days. Spike levels were at 50–200% of the native levels. All recoveries were within 90–110%, meeting the SMPR.

Table 5 shows the SRM 1849a results during the SLV. This SRM was tested 16 times with excellent precision. The mean results for Cr and Se were within the certified range, whereas Mo was just barely low. The subsequent collaborative study means (one analysis in duplicate from each of the eight laboratories using five different ICP/MS instrument models) were almost identical to those from the Abbott SLV, including the Mo value obtained.

Data for linearity and LOQ were obtained during the SLV, but these figures of merit have to be proven for a given instrument setup/model in the same way that the participating laboratories did the prework for the MLT.

Table 7. Collaborative study individual results for Cr (mg/kg, as is)[a]

Lab No.	Adult milk powdered		Infant powdered hydrolyzed milk		Adult powdered low fat		Child powder		Infant elemental powdered		Adult RTF high protein		Adult RTF high fat	
1[a]	0.025	0.018	<PLOQ	<PLOQ	0.083	0.073	0.036	0.045	0.026	0.016	0.020	0.022	0.019	0.022
2	0.121[b]	0.237[c]	<PLOQ	<PLOQ	0.424	0.359	0.371[c]	0.254[c]	0.220[d]	0.150[d]	0.143	0.126	0.112[e]	0.108[e]
3	0.130	0.145	<PLOQ	<PLOQ	0.385	0.386	0.240	0.250	0.192	0.185	0.105	0.125	0.128	0.122
4	0.140	0.166[f]	<PLOQ	<PLOQ	0.427	0.430	0.273	0.274	0.205	0.200	0.117	0.119	0.135	0.131
5	0.154	0.156	<PLOQ	<PLOQ	0.448	0.438	0.278	0.288	0.230[g]	0.250	0.128	0.129	0.130	0.135
8	0.121	0.124	<PLOQ	<PLOQ	0.438	0.474	0.293	0.272	0.218	0.197	0.125	0.125	0.126	0.130
9	0.172	0.168	<PLOQ	<PLOQ	0.437	0.447	0.331	0.286	0.213	0.229	0.135	0.126	0.127	0.127
10	0.194[h]	0.165	<PLOQ	<PLOQ	0.441	0.470	0.299	0.326	0.266	0.273	0.128	0.108	0.127	0.127
11	0.137	0.138	<PLOQ	<PLOQ	0.427	0.432	0.266	0.268	0.200	0.206	0.111	0.116	0.126	0.125
SLV ($n = 6$)	0.142		<PLOQ		0.428		0.272		0.215		0.130		0.141	

[a] Other rejected data are indicated by footnotes below. The value in each cell is the mean of the duplicate analyses required by the method. The paired results are the blind duplicates tested during the study.

[b] Rejected by Cochran's test; if not excluded RSD_R rises from 12.1 to 22.4%; if just the 0.237 value is rejected (so that eight laboratories have representative data), RSD_R is 12.8%.

[c] Rejected by Cochran's test; if not excluded RSD_R rises from 9.2 to 11.7%; if just the 0.371 value is rejected (so that eight laboratories have representative data), RSD_R is 9.3%.

[d] Rejected by Cochran's test; if not excluded RSD_R rises from 13.4 to 15.0%; if just the 0.150 value is rejected (so that eight laboratories have representative data), RSD_R is 12.7%.

[e] Although rejected by Single Grubbs' test these data points were kept in the final statistical analysis, as the data appear extraordinarily tight; if excluded, the RSD_R falls to 2.9% and the HorRat to 0.13 for this product.

[f] Rejected, 23% duplicate RSD; no retest result supplied.

[g] Rejected, 13% duplicate precision; no retest result supplied.

[h] Rejected, 15% duplicate RSD; no retest result supplied.

Table 8. Collaborative study raw individual results for Se (mg/kg, as is)[a]

Lab No.	Adult milk powdered		Infant powdered hydrolyzed milk		Adult powdered low fat		Child powder		Infant elemental powdered		Adult RTF high protein		Adult RTF high fat	
1[a]	0.182	0.203	0.183	0.182	0.208	0.211	0.193	0.210	0.123	0.112	0.045	0.073	0.087	0.098
2	0.236	0.215	0.227	0.244	0.304	0.291	0.214	0.205	0.210	0.220	0.109	0.107	0.135	0.143
3	0.225	0.230	0.234	0.238	0.299[b]	0.245[b]	0.235	0.250	0.253	0.252	0.107	0.0982[c]	0.129[d]	0.151[d]
4	0.213	0.209	0.235	0.229	0.249	0.254	0.215	0.204	0.200	0.203	0.0893	0.0913	0.138	0.135
5	0.247	0.237	0.240	0.269[e]	0.273	0.290	0.230	0.275[f]	0.213[g]	0.266g	0.0969	0.0989	0.129	0.137
8	0.227	0.232	0.243	0.247	0.273	0.273	0.218	0.222	0.206	0.216	0.0957	0.0919	0.134	0.134
9	0.235	0.230	0.234[h]	0.230	0.282	0.254[i]	0.219[j]	0.244	0.225	0.224	0.100	0.106	0.135[k]	0.135
10	0.204[l]	0.252[l]	0.237	0.231	0.245	0.256	0.195	0.215	0.216	0.199	0.0854	0.0849[m]	0.122	0.125
11	0.215	0.215	0.236	0.235	0.263	0.261	0.210	0.207	0.201	0.202	0.0914	0.0913	0.136	0.136
SLV (n = 6)	0.225		0.245		0.272		0.220		0.214		0.0961		0.140	

[a] Other rejected data are indicated by footnotes below. The value in each cell is the mean of the duplicate analyses required by the method. The paired results are the blind duplicates tested during the study.

[b] Although rejected by Cochran's test these data points were kept in the final statistical analysis, as the data appear extraordinarily tight; if excluded, the RSD_R falls to from 7.2 to 6.8%, and the HorRat from 0.37 to 0.35 for this product.

[c] Rejected, 8.4% duplicate precision, no retest result supplied.

[d] Although rejected by Cochran's test these data points were kept in the final statistical analysis, as the data appear extraordinarily tight; if excluded, the RSD_R falls to from 5.0 to 4.1%, and the HorRat from 0.23 to 0.19 for this product.

[e] Rejected, 7.5% duplicate precision, no retest result supplied.

[f] Rejected, 8.8% duplicate precision, no retest result supplied.

[g] Although rejected by Cochran's test these data points were kept in the final statistical analysis as a conservative measure; if excluded, the RSD_R falls to from 9.3 to 8.2%, and the HorRat from 0.46 to 0.41 for this product.

[h] Rejected, 15.1% duplicate precision, no retest result supplied.

[i] Rejected, 12.7% duplicate precision, no retest result supplied.

[j] Rejected, 8.4% duplicate precision, no retest result supplied.

[k] Rejected, 8.7% duplicate precision, no retest result supplied.

[l] Although rejected by Cochran's test these data points were kept in the final statistical analysis, as the data appear extraordinarily tight; if excluded, the RSD_R falls to from 6.1 to 5.2%, and the HorRat from 0.31 to 0.26 for this product.

[m] Rejected, 8.3% duplicate precision, no retest result supplied.

Table 9. Collaborative study individual results for Mo (mg/kg, as is)[a]

Lab No.	Adult milk powdered		Infant powdered hydrolyzed milk		Adult powdered low fat		Child powder		Infant elemental powdered		Adult RTF high protein		Adult RTF high fat	
1[a]	0.273	0.271	0.154	0.158	0.535	0.517	0.234	0.234	0.139	0.131	0.161	0.155	0.205	0.194
2	0.317[b]	0.366[b]	0.217[c]	0.194[c]	0.633	0.618	0.304[d]	0.272[d]	0.173	0.167	0.163	0.161	0.210	0.210
3	0.375	0.383	0.222	0.220	0.598	0.597	0.267	0.255	0.204	0.211	0.205[e]	0.203[e]	0.207	0.202
4	0.309	0.311	0.199	0.190	0.566	0.580	0.273	0.275	0.183	0.177	0.157	0.159	0.211	0.204
5	0.321	0.320	0.206	0.204[f]	0.606	0.592	0.290	0.280	0.185	0.188	0.164	0.166	0.203	0.203
8	0.301	0.304	0.192	0.192	0.564	0.586	0.272	0.269	0.171	0.170	0.154	0.155	0.195	0.192
9	0.312	0.314	0.195	0.195	0.583	0.592	0.275	0.275	0.169	0.172	0.157	0.155	0.193	0.196
10	0.341	0.334	0.225	0.228	0.592	0.593	0.299	0.289	0.199	0.203	0.160	0.156	0.195	0.195
11	0.327	0.330	0.207	0.211	0.613	0.599	0.288	0.287	0.181	0.181	0.167	0.167	0.213	0.213
SLV (n = 6)	0.294		0.184		0.565		0.261		0.160		0.154		0.193	

[a] Other rejected data are indicated by footnotes below. The value in each cell is the mean of the duplicate analyses required by the method. The paired results are the blind duplicates tested during the study.

[b] Rejected by Cochran's test; if not excluded (to maintain eight laboratories' data) RSD_R is unchanged at 7.9%.

[c] Although rejected by Cochran's test these data points were kept in the final statistical analysis, as the paired data appear extraordinarily tight; if excluded, the RSD_R remains essentially unchanged.

[d] Although rejected by Cochran's test these data points were kept in the final statistical analysis, as the data appear extraordinarily tight; if excluded, the RSD_R falls to from 4.6 to 4.3%, and the HorRat from 0.24 to 0.22 for this product.

[e] Rejected by Single Grubbs' test; if not excluded (to maintain eight laboratories' data) RSD_R rises from 3.0 to 9.0%.

[f] Rejected, 6.4% duplicate precision.

Collaborative Study Results and Discussion

The key precision performance metrics of the multilaboratory study per sample matrix are summarized in Table 6. The RSD_r derived from analysis of the blinded duplicates was roughly the same as for the known duplicates (raw data not shown from the participating laboratories, but the RSD_r obtained is consistent with the intermediate precision data from the SLV shown in Table 3). For instance, none of the seven matrixes produced an RSD_r higher than the method's 10% RSD duplicate criterion for Cr, 7% for Se, or 5% for Mo. Note, however, that repeatability of Se for three of the seven matrixes was between 5 and 7%, further justification for the change of this QC criterion to 7%. In terms of the repeatability SMPR, two matrixes had RSD_r of >5% for Cr, as did three matrixes for Se. The highest RSD_r observed for the blinded duplicates was 7.0%, and in this case, as well as the other four cases, the corresponding reproducibility was only slightly higher.

The RSD_R of method **2011.19** for each matrix was, on the average, about half of the SMPR of 15%. HorRat were similarly low, averaging 0.46 for Cr, 0.27 for Mo, and 0.32 for Se. The authors' opinion is that the RSD_R expected from this study is a function of how far above the instrument quantification limit we are at the determination stage, not of the absolute level of the analyte. Methods with good sensitivity, good linearity over the calibration range, and adequate required system suitability should be able to produce comparable reproducibility at the low ppb level, and this appears to be supported by other SPIFAN MLT studies (publications in progress).

The individual sample results submitted by each laboratory are given in Tables 7–9. Each value given is the mean of known duplicates, prepared per the method. Then, the blinded duplicate results are shown for each participating laboratory, for each matrix. The footnotes indicate which samples were rejected, either by the method's QC criteria, or by the AOAC-supplied statistical package (5). The laboratories could have analyzed new samples and obtained data to replace the rejected results, but there was not enough time to do so, or perhaps they did not realize this was an option. Although there were five cases in which both blind duplicate samples were rejected (thus Table 6 records the number of laboratories as seven for that matrix), the footnotes indicate that retaining the data would keep the RSD_R under 15% in all but one case. The data in Tables 7–9 also indicate why Laboratory 1 data were totally excluded from the study; except for a few Mo results, its data were significantly lower than that of any other laboratory across the board. Also, Laboratory 1 stood out as having the most problems with sensitivity and linearity (Table 1), and perhaps contamination was an issue due to the number of (known) duplicate failures for Cr (Table 2). It may be a coincidence, but that laboratory was using the oldest ICP/MS instrument, a PerkinElmer ELAN DRC-e, which may not have had the capability to do the required collisional/reaction chemistry to eliminate low-mass interferences.

Comments about the performance of the method were requested. One laboratory pointed out that the 10% powder reconstitution in the method was different than the 11.1% reconstitution recommended by SPIFAN and proceeded to use the latter (it made no discernible difference). Another comment was that the ICP/MS instrument model DRC-e could not use ammonia gas for Se determination, which may be the reason for Laboratory 1's exclusion from this study.

Conclusions

AOAC Method **2011.19** was successfully studied in collaboration by eight laboratories using multiple ICP/MS instrument models and testing a variety of infant, pediatric, and adult nutritional matrixes. The method demonstrated acceptable repeatability and reproducibility and met the SPIFAN SMPRs for reproducibility for all seven matrixes analyzed.

Recommendation

The multilaboratory collaborative study data were summarized and presented to the AOAC ERP in September 2014. After reviewing the data, the AOAC ERP voted to move AOAC **2011.19** to Final Action status, and the method was approved by the AOAC Official Methods Board as a Final Action method (6).

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INFANT FORMULA AND ADULT NUTRITIONALS

Analysis of Nucleotide 5′-Monophosphates in Infant Formulas by HPLC-UV: Collaborative Study, Final Action 2011.20

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A collaborative study was conducted on AOAC First Action Method 2011.20: 5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula. After the successful analysis of National Institute of Standards and Technology (NIST) 1849a Standard Reference Material (SRM) as a practice sample, 12 laboratories participated in the analysis of duplicate samples of six different infant formula products. The samples were dissolved in high-salt solution to inhibit protein and fat interactions, with the nucleotides [uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-monophosphate (CMP)] separated from the sample matrix by strong-anion exchange SPE, followed by chromatographic analysis using a C_{18} stationary phase with gradient elution, UV detection, and quantitation by an internal standard technique using thymidine 5′-monophosphate. For nucleotide-supplemented products, precision is within the *Standard Method Performance Requirements*^{SM} (SMPR) 2011.008 target reproducibility limit of ≤11%, with the reproducibility RSD (RSD_R) estimated at 7.1–8.7% for CMP, 7.9–9.0% for UMP, 2.8–7.7% for GMP, 5.5–10.3% for IMP, and 2.7–6.2% for AMP, and Horwitz ratio (HorRat) values of 0.9–1.0 for CMP, 0.9–1.0 for UMP, 0.3–0.7 for GMP, 0.6–1.0 for IMP, and 0.3–0.7 for AMP.

Nucleotides and nucleosides play important roles in cellular function as precursors to nucleic acids, as intermediaries in the transfer of chemical energy, and as critical components of coenzymes involved in carbohydrate, lipid, and protein metabolism. Although nucleotides are not essential dietary components as they can be synthesized de novo, they may be conditionally essential when the endogenous supply is insufficient, such as during periods of rapid neonatal growth. In recognition of their nutritional importance, infant formulas are increasingly supplemented with nucleotides. As neonates are dependent on a single dietary source for an extensive period, it is important that reliable analytical methods be available to accurately estimate the nucleotide content in infant formulas (1).

In view of the absence of an internationally accepted analytical method, nucleotides were identified by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) as a priority for which a reference method was urgently needed. The SPIFAN Nucleotides Working Group developed *Standard Method Performance Requirements*^{SM} (SMPR, 2011.008) for assessing merits of proposed nucleotide methods and established reproducibility limits of ≤11% in the range of 1–5 mg/hg reconstituted product, and ≤16% for 0.1 mg/hg reconstituted product.

We previously developed and performed a single-laboratory validation (SLV) study on an HPLC-UV method that incorporated SPE and internal standardization for the routine estimation of nucleotide 5'-monophosphates in milk and pediatric products (2). In September 2011, this HPLC-UV method was reviewed by an AOAC expert review panel (ERP) and, based on published SLV data, was approved for Official First Action status as AOAC Method **2011.20** (3, 4). The method subsequently underwent a comprehensive SLV study using a set of infant formula and adult nutritional products (SPIFAN Kit) that were selected as a representative subsample of the wide range of commercially available products, and the results were compared with the SMPR (5, 6). This SLV study was approved by the ERP in June 2012, and the method was recommended to advance to collaborative study for evaluation of reproducibility.

Collaborative Study

Although 19 laboratories initially indicated their interest to take part in this study, a number later withdrew primarily because of the timing of the study and difficulties with importation of the samples. Participating laboratories included those representing regulatory agencies, infant formula manufacturers, contract analytical services, and food research institutes. Prior to commencement of the study, each collaborator

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The method was approved by the AOAC *Official Methods Board* as Final Action. *See* "Standards News," (2014) *Inside Laboratory Management*, November/December 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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received a detailed study protocol to allow familiarization with the technique and an opportunity to communicate any difficulties. The NIST 1849a (National Institute of Standards and Technology, Gaithersburg, MD) Standard Reference Material (SRM) was selected as a practice sample to allow the laboratories to begin preliminary method evaluation. The distribution of the samples for this collaborative study was complicated because of the implementation of strict importation regulations by many countries; ultimately, only 12 laboratories from five countries were able to participate.

The SPIFAN Kit was unsuitable for use in this collaborative study because few of the included products were fortified with nucleotides; therefore, alternative sources of samples were required. Infant formula products (lactose-free, starch-based, hydrolysate-based, soy-based, and two whey-based) were sourced from manufacturing sites in Europe for subsampling and distribution, and each was pooled, mixed, subsampled into duplicate sachets (10 coded as blind-coded duplicates, two uncoded as a duplicate), sealed, and dispatched to the participating laboratories. The starch-based sample was uncoded because of the need for special handling during sample preparation. With the exception of the soy-based infant formula, all products had been supplemented with nucleotides during their manufacture.

Homogeneity of the nucleotides dispersed in the samples was assessed by replicate analyses of test samples from separate sachets (n = 5). Statistical analysis was on the basis of a paired t-test to establish significant difference between results obtained from different sachets. No bias was found between any sachets for any of the nucleotides, and the precision obtained was that expected for the concentration levels in these products (data not shown). On this basis, the samples were deemed to be fit for use in the collaborative study.

Upon completion of analysis of the samples, the collaborators were required to submit raw data as sample weights, UV absorbances of standard solutions, and peak areas for standards and samples, as well as the final results of nucleotide concentrations in the samples. Participants were also invited to add any relevant comments based on their experience in the use of the method.

All data were statistically analyzed using the AOAC protocol for overall mean, intralaboratory repeatability (S_r), repeatability RSD (RSD_r), interlaboratory reproducibility (S_R), reproducibility RSD (RSD_R), and Horwitz ratio (HorRat; 7). Cochran (P = 0.025, one-tail) and Grubbs (single and double, P = 0.025, two-tail) tests were utilized to determine outliers.

The method protocol sent to the collaborating laboratories was as described in AOAC First Action Method **2011.20**, with minor modifications to the nucleotide extinction coefficients (6) and to the sample preparation for starch-based products, based upon recommendations made by the ERP.

AOAC Official Method 2011.20
5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula
HPLC-UV
First Action 2011
Final Action 2015

(Applicable to the determination of nucleotide 5′-monophosphates in infant formula and adult/pediatric nutritional formula.)

Caution: Refer to the material safety data sheets for all chemicals prior to use. Use all appropriate personal protective equipment and follow good laboratory practices.

A. Principle

The sample is dissolved in high-salt solution to inhibit protein and fat interactions. The 5′-mononucleotides—uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-monophosphate (CMP)—are separated from the sample matrix by strong-anion exchange SPE, followed by chromatographic analysis using a C_{18} stationary phase with gradient elution, UV detection, and quantitation by an internal standard (IS) technique using thymidine 5′-monophosphate (TMP).

B. Apparatus

(**a**) *HPLC system*.—Equipped with pump, sample injector unit with a 50 µL injection loop, degasser unit, column oven, and photodiode array detector.

(**b**) *C_{18} column*.—Gemini C_{18}, 5 µm, 4.6 × 250 mm (Phenomenex, Torrance, CA) or equivalent.

(**c**) *Spectrophotometer*.—Capable of digital readout to 3 decimal places.

(**d**) *pH meter*.

(**e**) *Centrifuge*.

(**f**) *Amicon ultra centrifuge tubes*.—MWCO 3k, 4 mL (Millipore-Carrigtwohill, Co. Cork, Ireland) or equivalent.

(**g**) *Polypropylene centrifuge tubes*.—50 mL.

(**h**) *Disposable syringes*.—3 mL.

(**i**) *Syringe filters*.—0.2 µm with cellulose acetate membranes.

(**j**) *SPE vacuum manifold*.

(**k**) *Chromabond SB polypropylene strong-anion exchange SPE cartridges*.—6 mL × 1000 mg (Macherey-Nagel, Düren, Germany) or equivalent.

(**l**) *Filter membranes*.—0.45 µm nylon.

C. Reagents

(**a**) *Standards*.—Should be ≥99% pure (Sigma, St. Louis, MO, or equivalent). Nucleotide sodium salts or sodium salt hydrates may be substituted if free acid forms are not readily available.

(*1*) *TMP*.—CAS No. 365-07-1.

(*2*) *AMP*.—CAS No. 61-19-8.

(*3*) *CMP*.—CAS No. 63-37-6.

(*4*) *GMP*.—CAS No. 85-32-5.

(*5*) *IMP*.—CAS No. 131-99-7.

(*6*) *UMP*.—CAS No. 58-97-9.

(**b**) *Potassium bromide (KBr)*.

(**c**) *Potassium dihydrogen phosphate (KH_2PO_4)*.

(**d**) *Orthophosphoric acid (H_3PO_4)*.

(**e**) *Potassium hydroxide (KOH)*.

(**f**) *Ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA)*.

Table 2011.20A. UV absorbance maxima and extinction coefficients for nucleotide 5′-monophosphates

Nucleotide 5′-monophosphate	λ_{max}, nm	$E^{1\%}_{1cm}$
AMP	257	428.6
CMP	280	390.9
GMP	254	392.0
IMP	249	356.5
UMP	262	312.7
TMP	267	288.5

(**g**) *Sodium chloride (NaCl)*.

(**h**) *Methanol (MeOH)*.

(**i**) *Water*.—Purified with resistivity ≥18 MΩ.

D. Reagent Preparation

(**a**) *Standardizing buffer (KH_2PO_4, 0.25 M, pH 3.5)*.—Dissolve 34.0 g KH_2PO_4 in 900 mL water and adjust pH to 3.5 with H_3PO_4. Dilute to 1 L.

(**b**) *Extraction solution (NaCl 1 M, EDTA 4 mM)*.—Dissolve 58.5 g NaCl and 1.5 g EDTA in 1 L water.

(**c**) *Wash solution (KBr, 0.3 M)*.—Dissolve 3.6 g KBr in 100 mL water.

(**d**) *Eluent solution (KH_2PO_4, 0.5 M, pH 3.0)*.—Dissolve 6.8 g KH_2PO_4 in 90 mL water and adjust pH to 3.0 with H_3PO_4. Dilute to 100 mL.

(**e**) *Mobile phase A (KH_2PO_4, 10 mM, pH 5.6)*.—Dissolve 1.4 g KH_2PO_4 in 900 mL water and adjust pH to 5.6 with KOH solution (10%, w/v). Dilute to 1 L with water. Make daily as microbial growth often occurs at room temperature in phosphate buffers that contain little or no organic solvent.

(**f**) *Mobile phase B*.—100% MeOH.

E. Standard Preparation

See Table **2011.20A** for the UV absorbance maxima and extinction coefficients for nucleotide 5′-monophosphates.

(**a**) *Stock standard solutions (approximately 1 mg/mL)*.—Accurately weigh approximately 50 mg each nucleotide 5′-monophosphate into separate 50 mL volumetric flasks. Add 40 mL water, mix until dissolved, and fill to volume with water.

(**b**) *Purity standard solutions*.—Pipet 1.0 mL each stock standard into separate 50 mL volumetric flasks, make to volume with standardizing buffer (KH_2PO_4, 0.25 M, pH 3.5), and measure absorbance at the appropriate λ_{max} to determine the concentration of each nucleotide stock standard.

Table 2011.20B. Nominal concentrations of calibration standards

Calibration solution	Concn of AMP, CMP, GMP, IMP, UMP, µg/mL	Concn of TMP, µg/mL
1	0.4	3.2
2	0.8	3.2
3	3.2	3.2
4	8.0	3.2

Table 2011.20C. Gradient procedure for chromatographic separation

Time, min	Flow rate, mL/min	Mobile phase A, %	Mobile phase B, %
0	0.6	100	0
25	0.6	80	20
26	0.6	100	0
40	0.6	100	0

(**c**) *Internal standard solution (approximately 80 µg/mL)*.—Dilute 4 mL TMP stock standard in 50 mL water.

(**d**) *Working standard solution (approximately 40 µg/mL)*.—Pipet 2 mL each stock standard (AMP, CMP, GMP, IMP, and UMP) into a single 50 mL volumetric flask and make to volume with water.

(**e**) *Calibration standard solutions*.—*See* Table **2011.20B** for nominal nucleotide concentrations of the calibration standard solutions.

(*1*) *Calibration standard 1*.—Pipet 0.25 mL working standard and 1 mL internal standard into a 25 mL volumetric flask and make to volume with water.

(*2*) *Calibration standard 2*.—Pipet 0.5 mL working standard and 1 mL internal standard into a 25 mL volumetric flask and make to volume with water.

(*3*) *Calibration standard 3*.—Pipet 2 mL working standard and 1 mL internal standard into a 25 mL volumetric flask and make to volume with water.

(*4*) *Calibration standard 4*.—Pipet 5 mL working standard and 1 mL internal standard into a 25 mL volumetric flask and make to volume with water.

F. Sample Preparation

(**a**) Shake or mix sample container prior to opening.

(**b**) Accurately weigh approximately 1 g powder or 10 mL ready-to-feed/liquid milk infant formula/adult nutritional product into a 50 mL centrifuge tube.

(**c**) Add 30 mL extraction solution (NaCl 1 M, EDTA 4 mM).

(**d**) Add 1.0 mL TMP IS (approximately 80 µg/mL).

(**e**) Cap the tube and vortex mix until powder dissolved.

(**f**) Allow sample to stand for 10 min to ensure complete hydration.

(**g**) Dilute to a final volume of 50 mL with water.

(**h**) Cap the tube and vortex mix.

(**i**) For starch-based products, transfer 2 × 4 mL prepared sample to two separate ultra centrifuge tubes and centrifuge at 3500 × *g* for 60 min, and then pool filtrates from both tubes.

G. Extraction

Throughout the extraction procedure, do not let the cartridge run dry but drain to the top of the cartridge bed only. When draining the cartridge, the flow rate should be <2 mL/min.

(**a**) For each sample, place a single SPE cartridge on a vacuum manifold.

(**b**) Condition the columns by adding 4 mL methanol and draining to the top of the cartridge bed, followed by adding two aliquots of water (5 mL each) and draining to the top of the cartridge bed.

(**c**) Load the cartridge with sample solution (4 mL) and drain to the top of the cartridge bed.

(**d**) Wash the cartridge to remove interferences with wash solution (KBr, 0.3 M, 4 mL) and drain to the top of the cartridge bed.

(**e**) Place a sample collection tube in the SPE manifold.

(**f**) Elute the nucleotides with eluent solution (KH_2PO_4, 0.5 M, pH 3.0, 4 mL) into a sample collection tube and completely drain the cartridge.

(**g**) Filter an aliquot (approximately 2 mL) eluent through a 0.2 µm syringe filter into an autosampler vial.

H. Chromatography

(**a**) Form gradients by low pressure mixing of the two mobile phases, A and B, with separation of nucleotides achieved using the procedure shown in Table **2011.20C**.

(**b**) Acquire spectral data between 210 and 300 nm using the photodiode array detector with chromatograms monitored at the specified wavelengths below for quantitation.

(*1*) IMP wavelength at 250 nm.

(*2*) AMP, GMP, and TMP wavelengths at 260 nm.

(*3*) CMP and UMP wavelengths at 270 nm.

(**c**) Set column oven to 40°C.

I. Calculations

(**a**) Concentration of nucleotide in stock standard (SS):

$$\text{SS, µg/mL} = \frac{wtSS}{50} \times \frac{PS\%}{100} \times 10^3$$

where wtSS = weight of nucleotide in stock standard (mg), 50 = total volume of SS (mL), 10^3 = concentration conversion (mg/mL to µg/mL), PS% = percent purity, and 100 = mass conversion (% to decimal).

(**b**) Percentage purity of each nucleotide (as free acid) in purity standard (PS):

$$\text{Purity, \%} = \frac{\text{Abs}_{\lambda max}}{E^{1\%}_{1cm}} \times \frac{50}{\text{wtSS}} \times \frac{50}{1} \times 1000$$

where $\text{Abs}_{\lambda max}$ = UV absorbance at maximum wavelength, $E^{1\%}_{1cm}$ = extinction coefficient for nucleotide, wtSS = weight of nucleotide in stock standard (mg), 50 = total volume of stock standard (mL), 50 = total volume of purity standard (mL), 1 = volume of stock standard added to purity standard (mL), and 1000 = mass conversion from mg to g.

(**c**) Concentration of TMP in IS:

$$\text{IS, µg/mL} = \text{SS} \times \frac{4}{50}$$

where SS = concentration of TMP in stock standard (µg/mL), 4 = volume of TMP stock standard in IS (mL), and 50 = total volume of IS (mL).

(**d**) Concentration of nucleotides in working standard (WS):

$$\text{WS, µg/mL} = \text{SS} \times \frac{2}{50}$$

where SS = concentration of nucleotide in stock standard (µg/mL), 2 = volume of nucleotide stock standard in working standard (mL), and 50 = total volume of working standard (mL).

(**e**) Concentration of TMP in calibration standards (CS):

$$\text{CS, µg/mL} = \text{IS} \times \frac{1}{25}$$

where IS = concentration of nucleotide in IS (µg/mL), 1 = volume of IS in calibration standard (mL), and 25 = total volume of calibration standard (mL).

(**f**) Concentration of nucleotides in calibration standard (CS):

$$\text{CS, µg/mL} = \text{WS} \times \frac{V_{WS}}{25}$$

where WS = concentration of nucleotide in working standard (µg/mL), V_{WS} = volume of working standard in CS (mL), and 25 = total volume of CS (mL).

(**g**) Determine the linear regression curve for the ratio of peak areas (nucleotide/TMP; y-axis) versus the ratio of concentrations (nucleotide/TMP; x-axis) for CSs and calculate the slope with the y-intercept forced through 0.

(**h**) Interpolate the nucleotide contents in unknown samples from this calibration curve.

(*1*) For powders:

$$\text{Nucleotide, mg/hg} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{W_S} \times \frac{100}{1000}$$

(*2*) For ready-to-feed liquids:

$$\text{Nucleotide, mg/dL} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{V_S} \times \frac{100}{1000}$$

where A_{NT} = nucleotide peak area in sample, A_{IS} = TMP peak area in sample, L = linear regression slope of calibration curve, C_{IS} = concentration of IS added to sample (µg/mL), V_{IS} = volume of IS added to sample (mL), W_S = weight of sample (g), 1000 = mass conversion of result (µg to mg), V_S = volume of sample (mL), and 100 = mass or volume conversion of result (g to 100 g; mL to 100 mL).

J. Data Handling

Report results in mg/hg or mg/dL to 1 decimal place.

Results and Discussion

The initial phase of method evaluation within the participating laboratories involved the analysis of a practice sample. The NIST 1849a SRM was selected for this purpose for a number of reasons: (*1*) as it was readily available in most laboratories, the method setup and evaluation could commence without receipt of shipped samples; (*2*) participants could evaluate method implementation in their laboratory against certified values; and (*3*) it provided additional confidence that there was no significant bias in method performance among all participants.

Precision and bias were evaluated for NIST 1849a practice samples as defined by the AOAC ERP (8). All participating laboratories provided acceptable data for the practice sample (Table 1) and, when the test sample set had been received, participants could begin the analysis at their earliest convenience.

Upon completion of the analyses, each participant reported the results accompanied by calibration regression parameters and a description of any method deviations. All 12 laboratories returned acceptable standard calibration parameters based on linear regression correlation coefficients (r^2: 0.9971–1.0000). The analytical results submitted by the participants were

collated (Tables 2–6) and statistically analyzed (Tables 7–11). In some instances, statistical outliers were identified, but, where deemed to be reasonable to do so, these were retained in the data set for calculation of the method precision.

As the soy-based infant formula was not fortified with nucleotides and contained endogenous levels only, the precision for this sample was poor, as expected at concentrations near or below the method detection limit (2). The mean nucleotide concentrations in the supplemented infant formula powders were in the ranges 5.4–11.4 mg/hg for CMP, 3.5–4.2 mg/hg for UMP, 1.1–1.7 mg/hg for GMP, 1.7–2.5 mg/hg for IMP, and 3.3–4.7 mg/hg for AMP. The RSD_r values obtained were in the ranges 1.1–2.7% for CMP, 1.5–5.4% for UMP, 1.6–3.9% for GMP, 1.4–2.8% for IMP, and 1.3–3.9% for AMP. The RSD_R values obtained were in the ranges 7.1–8.7% for CMP, 7.9–9.0% for UMP, 2.8–7.7% for GMP, 5.5–10.3% for IMP, and 2.7–6.2% for AMP. In all instances of nucleotide-supplemented infant formulas, the repeatability and the reproducibility were within limits set in the SMPR for nucleotides (6). Acceptable reproducibility was also demonstrated, with HorRat values for the method in the ranges 0.9–1.0 for CMP, 0.9–1.0 for UMP, 0.3–0.7 for GMP, 0.6–1.0 for IMP, and 0.3–0.7 for AMP (recommended range 0.5–2.0; 9).

A summary of each laboratory's performance was sent to participants, along with an invitation to make comments on the performance of the method in their laboratory. In general, comments were positive with respect to the use of the method and intralaboratory performance. Laboratory 3 recommended that EDTA used be standardized to the salt form. It was noted by Laboratory 5 that, if the pH of the mobile phase was higher by >0.3 pH units, the elution sequence changed for AMP and TMP. Some concerns were expressed by Laboratory 7 regarding the value of the extinction coefficient for CMP. Follow-up work was undertaken, and the extinction coefficient used for CMP was verified by Laboratory 7 after an investigation with the supplier of the standard. Laboratory 9 recommended a 5 min centrifugation of the samples prior to the SPE step. Laboratory 10 suggested adding a reduced amount of extract to the SPE cartridge to make the method more applicable to various product matrixes.

The method has demonstrated its compliance with the applicability statement of SMPR 2011.008 (6), and it has been shown in this collaborative study to be suitable for the analysis of nucleotides in a wide range of supplemented infant formulas. The method has been demonstrated to be unsuitable for samples containing endogenous nucleotide levels only. Nucleosides are an optional nutrient defined by the SMPR and are not determined with this method. Although the method may be applicable to adult nutritional products, such products are generally not fortified with nucleotides because they are not considered to be an essential dietary nutrient for adults.

Conclusions

A collaborative study of the AOAC First Action **2011.20** HPLC-UV method for the analysis of nucleotides in infant formula was undertaken. The method was applied to a number of different infant formula matrixes and demonstrated acceptable reproducibility precision for nucleotide-supplemented infant formulas.

Recommendation

A study report summarizing the outcomes of this collaborative study was submitted with the recommendation that AOAC First Action Method **2011.20** be accepted as a SPIFAN-endorsed

Table 1. Bias and precision results for NIST 1849a practice sample

Statistic	CMP[a]	UMP[a]	GMP[a]	IMP[a]	AMP[a]
Total number of laboratories	12	12	12	12	12
Total number of replicates (*n*)	24	24	24	24	24
Mean ($\bar{x}$)[b]	28.1	11.8	15.1	0	10.9
Certified value (μ)[b]	26.8	12.9	14.6	—[c]	10.5
Uncertainty (U_{CRV})[b]	2.9	1.5	1.1	—	0.53
Coverage factor (*k*)	2.57	2.57	2.57	—	2.57
Nominal bias, %	5.00	–8.90	3.20	—	3.50
Student's test-statistic (t_{Stat})	1.16	1.92	1.05	—	1.63
Degrees of freedom (*DF*)	5.6	5.4	6.1	—	7.3
P-value	0.30	0.11	0.34	—	0.15
Repeatability SD (SD_r)[b]	0.46	0.30	0.38	—	0.22
Reproducibility SD (SD_R)[b]	1.36	0.59	0.68	—	0.47
Repeatability RSD (RSD_r), %	1.6	2.5	2.5	—	2.1
Reproducibility RSD (RSD_R), %	4.8	5.0	4.5	—	4.4
Horwitz ratio (HorRat)	0.7	0.6	0.6	—	0.6

[a] CMP = Cytidine 5′-monophosphate, UMP = uridine 5′-monophosphate, GMP = guanosine 5′-monophosphate, IMP = inosine 5′-monophosphate, AMP = adenosine 5′-monophosphate.

[b] Concentration in mg/hg.

[c] — = Not applicable.

Table 2. Collaborative study data for CMP in infant formulas[a]

Lab No.	NIST 1849a		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[b]		Whey-based		Whey-based	
1	27.88	27.97	11.09	11.12	10.75	10.73	9.61	9.63	0.49	0.47	5.20	5.25	5.17	5.21
2	29.60	29.10	9.52	9.48	10.49	9.43	10.3	10.10	0.00	0.00	4.55	4.40	3.94[c]	3.56[c]
3	27.46	26.90	10.71	10.50	9.90	9.98	9.60	9.53	0.00	0.00	6.23	5.98	5.05	4.81
4	29.66	30.45	12.40	12.40	11.90	12.30	10.70	10.90	1.00	1.10	6.00	6.10	6.20	6.00
5	28.77	28.65	12.84	13.12	11.92	11.92	10.91	10.20	0.58	0.78	5.86[d]	6.41[d]	6.24	6.14
6	27.80	28.00	11.68	11.76	11.27	11.20	10.23	10.21	0.88	0.89	5.48	5.43	5.47	5.41
7	27.55	29.25	11.28	11.00	11.28	11.22	9.49	8.56	0.56	0.46	5.30	5.26	5.27	5.15
8	27.65	27.88	11.65	11.52	11.10	10.93	8.93	9.19	0.44	0.48	5.41	5.39	5.36	5.41
9	28.21	28.17	11.53	11.56	2.77[e]	3.08[e]	9.96	9.96	0.59	0.47	5.44	5.47	5.44	5.46
10	29.50	29.60	11.86	11.95	11.44	11.44	9.83	9.98	0.66	0.62	5.58	5.59	5.63	5.60
11	24.54	25.27	11.60	11.90	11.03	11.84	10.30	10.10	0.90	0.00	5.50	5.20	5.30	5.50
12	28.08	27.53	10.78	10.94	9.91	9.79	9.03	9.09	0.30	0.35	5.13	5.23	4.80	4.83

[a] Concentration in mg/hg.

[b] Product not fortified with CMP.

[c] Identified as Grubbs outlier; results removed from data set for statistical analysis.

[d] Identified as Cochran outlier; results kept in data set for statistical analysis.

[e] Problems were identified by participants; identified as Grubbs outlier; results excluded from data set for statistical analysis.

Table 3. Collaborative study data for UMP in infant formulas[a]

Lab. No.	NIST 1849a		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[b]		Whey-based		Whey-based	
1	11.75	11.58	3.94	3.95	4.03	4.14	4.30	4.22	0.29	0.32	3.47	3.51	3.60	3.60
2	11.30	11.10	3.75	3.83	3.91	3.78	4.00	3.70	0.00	0.00	3.25	3.26	3.26	3.11
3	10.59	10.51	3.27	3.26	3.14[c]	3.11[c]	3.59	3.38	0.00	0.00	2.97	2.90	2.70	3.11
4	12.14	12.00	4.00	4.00	3.90	3.90	4.40	4.40	0.20	0.20	3.60	3.60	3.60	3.80
5	10.91	11.29	3.51	3.34	3.44[d]	4.29[d]	4.11	4.31	0.19	0.00	3.78	3.93	3.85	3.78
6	11.60	11.80	4.32	4.30	4.23	4.24	4.56	4.51	0.28	0.25	3.87	3.84	3.83	3.87
7	12.26	12.36	3.93	4.00	4.02	3.89	4.25	4.47	0.32	0.28	3.70	3.56	3.49	3.73
8	12.04	12.57	3.66	3.80	4.04	3.77	3.63	3.58	0.38	0.38	3.64	3.66	3.69	3.60
9	12.25	11.79	3.97	4.03	1.19[e]	0.00[e]	4.33	4.28	0.35	0.22	3.58	3.55	3.67	3.64
10	11.80	11.90	3.84	3.88	3.91	3.93	4.51	4.11	0.22	0.23	3.53	3.59	3.60	3.61
11	12.05	12.45	3.50	3.80	3.73	3.91	4.00	3.70	0.00	0.00	3.00	3.00	3.10	3.10
12	11.50	12.59	4.28	4.05	3.93	4.17	4.53	4.60	0.27	0.29	3.75	3.87	3.78	3.89

[a] Concentration in mg/hg.

[b] Product not fortified with UMP.

[c] Identified as Cochran outlier; results kept in data set for statistical analysis.

[d] Identified as Grubbs outlier; results removed from data set for statistical analysis.

[e] Problems were identified by participants; identified as Grubbs outlier; results excluded from data set for statistical analysis.

Table 4. Collaborative study data for GMP in infant formulas[a]

Lab No.	NIST 1849a		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[b]		Whey-based		Whey-based	
1	14.31	14.40	1.39	1.39	1.59	1.60	1.33	1.35	0.26	0.32	1.01	1.01	0.99	1.02
2	15.80	15.50	1.50[c]	1.40[c]	1.78	1.78	1.40	1.40	0.00	0.00	1.06	1.05	0.70[d]	1.02[d]
3	15.15	14.86	1.44	1.40	1.63	1.63	1.60	1.58	0.00	0.00	1.13	1.13	1.12	1.17
4	14.77	15.36	1.50	1.50	1.70	1.70	1.40	1.40	0.00	0.00	1.10	1.10	1.10	1.10
5	14.92	14.93	1.45	1.45	1.67	1.69	1.44	1.33	0.25	0.41	1.07	1.09	1.08	1.02
6	14.60	14.70	1.40	1.45	1.63	1.60	1.48	1.39	0.23	0.25	1.06	1.03	1.05	1.05
7	13.97	15.63	1.43	1.40	1.65	1.60	1.25[c]	1.04[c]	0.23	0.20	0.98	0.98	0.91	1.01
8	14.68	14.84	1.45	1.46	1.67	1.62	1.33	1.34	0.37	0.54	1.06	1.05	1.05	1.06
9	15.12	15.08	1.47	1.45	0.00[e]	0.00[e]	1.41	1.40	0.29	0.3	1.05	1.08	1.08	1.09
10	15.30	15.30	1.48	1.50	1.72	1.79	1.36	1.41	0.36	0.35	1.07	1.06	1.08	1.06
11	16.73	16.69	1.50	1.50	1.78	1.75	1.40	1.40	0.00	0.00	1.10[c]	1.00[c]	1.00	1.10
12	14.60	14.41	1.42	1.40	1.64	1.58	1.39	1.38	0.49	0.45	1.03	1.00	1.02	1.04

[a] Concentration in mg/hg.

[b] Product not fortified with GMP.

[c] Identified as Cochran outlier; results kept in data set for statistical analysis.

[d] Identified as Grubbs outlier; results removed from data set for statistical analysis.

[e] Problems were identified by participants; identified as Grubbs outlier; results excluded from data set for statistical analysis.

Table 5. Collaborative study data for IMP in infant formulas[a]

Lab No.	NIST 1849a		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[b]		Whey-based[b]		Whey-based[b]	
1	0.00	0.00	1.52	1.53	1.51	1.49	2.32	2.34	0.21	0.13	0.03[c]	0.02[c]	0.02[c]	0.05[c]
2	0.00	0.00	1.87	1.82	1.82	1.84	2.50	2.50	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	1.64	1.63	1.58	1.59	2.46	2.44	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	1.70	1.70	1.70	1.70	2.95[c]	3.90[c]	0.00	0.00	0.00	0.00	0.00	0.00
5	0.57	0.32	1.77	1.73	2.05	2.07	2.52	2.54	0.29	0.60	0.00[d]	0.22[d]	0.00	0.00
6	0.00	0.00	1.58	1.59	1.55	1.56	2.37	2.35	0.16	0.13	0.00	0.00	0.00	0.00
7	0.00	0.00	1.46	1.54	1.47	1.44	2.40[d]	2.21[d]	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	1.63	1.61	1.61	1.61	2.38	2.42	0.22	0.26	0.00	0.00	0.00	0.00
9	0.00	0.00	1.61	1.63	0.00[e]	0.00[e]	2.39	2.41	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	1.66	1.68	1.63	1.63	2.38	2.43	0.13	0.13	0.00	0.00	0.00	0.00
11	0.00	0.00	1.80[d]	1.60[d]	1.80[d]	1.71[d]	2.50	2.50	0.00	0.00	0.00	0.00	0.00	0.00
12	0.00	0.00	1.60	1.59	1.62	1.59	2.78	2.74	0.89	0.76	0.00	0.00	0.35[c]	0.24[c]

[a] Concentration in mg/hg.

[b] Product not fortified with IMP.

[c] Identified as Grubbs outlier; results removed from data set for statistical analysis.

[d] Identified as Cochran outlier; results kept in data set for statistical analysis.

[e] Problems were identified by participants; identified as Grubbs outlier; results excluded from data set for statistical analysis.

Table 6. Collaborative study data for AMP in infant formulas[a]

Lab No.	NIST 1849a		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[b]		Whey-based		Whey-based	
1	10.69	10.75	3.33	3.32	3.45	3.48	4.96	4.95	0.57	0.97	3.69	3.69	3.68	3.70
2	11.80	11.60	3.27	3.09	3.53	3.52	5.10	4.70	0.71	1.00	3.15	3.06	2.58[c]	3.09[c]
3	11.15	10.89	3.29	3.32	3.38	3.42	4.79	4.76	0.00	0.00	3.35	3.52	3.41	3.35
4	11.05	11.23	3.40	3.40	3.60	3.70	5.10	5.20	0.60	0.60	3.70	3.60	3.70	3.70
5	10.84	11.22	3.52	3.45	3.56	3.79	4.88	4.89	0.41	0.59	3.48	3.52	3.52	3.49
6	10.70	10.70	3.35	3.35	3.52	3.46	4.75	4.72	0.44	0.43	3.46	3.40	3.43	3.40
7	9.59	10.42	3.32	3.28	3.59	3.58	4.57[c]	3.84[c]	0.51	0.50	3.64	3.51	3.63[c]	3.48[c]
8	10.82	10.90	3.43	3.41	3.55	3.42	4.32	4.51	0.74	0.86	3.42	3.39	3.36	3.39
9	10.34	10.28	3.26	3.27	0.00[d]	0.00[d]	4.67	4.65	0.63	0.61	3.39	3.39	3.35	3.32
10	11.30	11.30	3.42	3.43	3.58	3.54	4.79	5.01	0.86	0.88	3.68	3.71	3.73	3.72
11	11.25	10.79	3.40	3.30	3.56	3.75	5.10	4.70	0.00	0.00	3.60	3.50	3.60	3.60
12	10.69	10.59	3.24	3.27	3.46	3.42	4.79	4.77	0.54	0.55	3.67	3.70	3.30	3.43

[a] Concentration in mg/hg.

[b] Product not fortified with AMP.

[c] Identified as Cochran outlier; results kept in data set for statistical analysis.

[d] Identified as Grubbs outlier; results removed from data set for statistical analysis.

Table 7. Collaborative study results for CMP in infant formulas

Infant formula	Laboratories[a]	*n*[b]	Mean, mg/hg	S_r, mg/hg	S_R, mg/hg	RSD_r, %	RSD_R, %	HorRat
Lactose-free	12 (0)	24 (0)	11.42	0.12	0.89	1.1	7.8	1.0
Starch-based	11 (1)	22 (2)	10.99	0.30	0.81	2.7	7.4	0.9
Hydrolysate-based	12 (0)	24 (0)	9.72	0.26	0.69	2.7	7.1	0.9
Soy-based[c]	12 (0)	24 (0)	0.50	0.19	0.34	38.5	67.1	5.3
Whey-based	12 (0)	24 (0)	5.47	0.15	0.48	2.7	8.7	1.0
Whey-based	11 (1)	22 (2)	5.43	0.09	0.43	1.6	7.9	0.9

[a] Number of laboratories with results submitted (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Product not fortified with CMP.

Table 8. Collaborative study results for UMP in infant formulas

Infant formula	Laboratories[a]	n[b]	Mean, mg/hg	S_r, mg/hg	S_R, mg/hg	RSD_r, %	RSD_R, %	HorRat
Lactose-free	12 (0)	24 (0)	3.84	0.09	0.30	2.4	7.9	0.9
Starch-based	11 (1)	22 (2)	3.88	0.21	0.31	5.4	8.4	0.9
Hydrolysate-based	12 (0)	24 (0)	4.15	0.13	0.36	3.1	8.7	1.0
Soy-based[c]	12 (0)	24 (0)	0.19	0.05	0.14	25.0	72.0	5.0
Whey-based	12 (0)	24 (0)	3.52	0.05	0.31	1.5	8.8	0.9
Whey-based	12 (0)	24 (0)	3.54	0.11	0.32	3.2	9.0	1.0

[a] Number of laboratories with results submitted (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Product not fortified with UMP.

Table 9. Collaborative study results for GMP in infant formulas

Infant formula	Laboratories[a]	n[b]	Mean, mg/hg	S_r, mg/hg	S_R, mg/hg	RSD_r, %	RSD_R, %	HorRat
Lactose-free	12 (0)	24 (0)	1.45	0.03	0.04	1.8	2.8	0.3
Starch-based	11 (1)	22 (2)	1.67	0.03	0.07	1.6	4.2	0.4
Hydrolysate-based	12 (0)	24 (0)	1.38	0.05	0.11	3.9	7.7	0.7
Soy-based[c]	12 (0)	24 (0)	0.22	0.05	0.18	22.9	82.7	5.8
Whey-based	12 (0)	24 (0)	1.05	0.02	0.04	2.2	4.1	0.4
Whey-based	11 (0)	22 (2)	1.05	0.04	0.05	3.4	5.2	0.5

[a] Number of laboratories with results submitted (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Product not fortified with GMP.

Table 10. Collaborative study results for IMP in infant formulas

Infant formula	Laboratories[a]	n[b]	Mean, mg/hg	S_r, mg/hg	S_R, mg/hg	RSD_r, %	RSD_R, %	HorRat
Lactose-free	12 (0)	24 (0)	1.65	0.05	0.10	2.8	6.1	0.6
Starch-based	11 (1)	22 (2)	1.66	0.02	0.17	1.4	10.3	1.0
Hydrolysate-based	11 (1)	22 (2)	2.46	0.04	0.13	1.8	5.5	0.6
Soy-based[c]	12 (0)	24 (0)	0.16	0.07	0.25	43.7	156.2	10.5
Whey-based[c]	10 (2)	20 (4)	ND[d]	—[e]	—	—	—	—
Whey-based[c]	10 (2)	20 (4)	ND	—	—	—	—	—

[a] Number of laboratories with results submitted (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Product not fortified with IMP.

[d] ND = Not detected.

[e] — = Not applicable.

Table 11. Collaborative study results for AMP in infant formulas

Infant formula	Laboratories[a]	n[b]	Mean, mg/hg	S_r, mg/hg	S_R, mg/hg	RSD_r, %	RSD_R, %	HorRat
Lactose-free	12 (0)	24 (0)	3.34	0.05	0.09	1.4	2.7	0.3
Starch-based	11 (1)	22 (2)	3.54	0.08	0.11	2.1	3.0	0.3
Hydrolysate-based	12 (0)	24 (0)	4.73	0.19	0.30	3.9	6.2	0.7
Soy-based[c]	12 (0)	24 (0)	0.54	0.11	0.30	20.4	55.7	4.6
Whey-based	12 (0)	24 (0)	3.51	0.06	0.18	1.7	5.0	0.5
Whey-based	11 (1)	22 (2)	3.51	0.05	0.15	1.3	4.3	0.5

[a] Number of laboratories with results submitted (number of laboratories with data removed as outliers).

[b] Number of samples with results submitted (number of samples removed as outliers).

[c] Product not fortified with AMP.

AOAC Final Action Method. The AOAC ERP evaluated the collaborative study data in September 2014, and endorsed the recommendation, which was subsequently approved by the Official Methods Board in November 2014.

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Norman White, Abbott Laboratories, Columbus, OH

Bolong Wu, CAIQ, Beijing, China

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Vitamin E and Vitamin A in Infant Formula and Adult Nutritionals by Normal-Phase High-Performance Liquid Chromatography: Collaborative Study, Final Action 2012.10

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The main objective of the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) project is to establish international consensus methods for infant formula and adult nutritionals, which will benefit intermarket supply and dispute resolution. A collaborative study was conducted on AOAC First Action Method 2012.10 *Simultaneous Determination of 13-*cis *and All-*trans *Vitamin A Palmitate (Retinyl Palmitate), Vitamin A Acetate (Retinyl Acetate), and Total Vitamin E (α-Tocopherol and D-α-tocopherol acetate) in Infant Formula and Adult Nutritionals by Normal-Phase HPLC*. Fifteen laboratories from 11 countries participated in an interlaboratory study to determine 13-*cis* and all-*trans* vitamin A palmitate (retinyl palmitate), vitamin A acetate (retinyl acetate), and total vitamin E (α-tocopherol and D-α-tocopherol acetate) in infant formula and adult nutritionals by normal-phase HPLC and all laboratories returned valid data. Eighteen test portions of nine blind duplicates of a variety of infant formula and adult nutritional products were used in the study. The matrixes included milk-based and soy-based hydrolyzed protein as well as a low fat product. Each of the samples was prepared fresh and analyzed in singlicate. As the number of samples exceeded the recommended number to be prepared in a single day, analysis took place over 2 days running 12 samples on day one and 10 samples on day two. The reference standard stock was prepared once and the six-point curve diluted freshly on each day. Results obtained from all 15 laboratories are reported. The RSD_R for total vitamin A (palmitate or acetate) ranged from 6.51 to 22.61% and HorRat values ranged from 0.33 to 1.25. The RSD_R for total vitamin E (as tocopherol equivalents) ranged from 3.84 to 10.78% and HorRat values ranged from 0.27 to 1.04. Except for an adult low fat matrix which generated reproducibility RSD >40% for some isomers, most SPIFAN matrixes gave results within the acceptance criteria of <16% RSD as stated in the respective *Standard Method Performance Requirements.*

Received April 29, 2015.
The method was approved by the AOAC Official Methods Board as Final Action. *See* "Standards News," (2014) *Inside Laboratory Management*, November/December 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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Vitamin A (retinol) is an essential nutrient for normal vision, and teeth and bone formation. An inadequate intake of vitamin A causes xerophthalmia, resulting in blindness, stunted growth, and possible death. An overdose of vitamin A is damaging to infants and adults. Vitamin A can exist in several isomeric forms and as esters. Retinyl palmitate will isomerize under thermal and photochemical stress to a variety of *cis*-isomers, of which 13-*cis* is the most common and most active (75% of *trans*). Other isomers have reduced vitamin A activity. In this method, no distinction is made between the bioactivities of the isomers; instead, all are summed against the *trans* isomer to give the total vitamin A concentration.

Although vitamin E has been known since the 1920s, its functions have only recently been defined. The principle role of vitamin E is as an antioxidant, protecting many other biochemicals from damage by active oxygen and other free radicals. It works closely with vitamin C in this respect, particularly in cell membranes. Vitamin E has eight active forms which vary in methyl-substitution in the tocol ring (α-, β-, γ-, and δ-tocopherols) and in saturation of the side chain (α-, β-, γ-, and δ-tocotrienols). In food science, only α-tocopherol is usually considered, because it is the most active and most abundant vitamer.

At the AOAC INTERNATIONAL Annual Meeting on September 29, 2012, the AOAC Expert Review Panel (ERP) for the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Nutrient methods reviewed this method separately for vitamins A and E, including all available method validation data. Following the evaluation of the data for both methods, the ERP granted First Action status to both methods and recommended that a single method be published for the simultaneous determination of vitamin A palmitate, vitamin A acetate and total vitamin E (D-α-tocopherol and D-α-tocopherol acetate) in infant formula and adult nutritionals by normal-phase HPLC. Following the completion of a single-laboratory validation

(SLV) using SPIFAN matrixes (a selection of commercially available infant and adult nutritional formulas), AOAC First Action Method **2012.10** was published in the *Journal of AOAC INTERNATIONAL* in 2013 (1). Following the successful outcome of SLV, the method was chosen to go forward for multilaboratory testing (MLT; collaborative study) by the ERP at the annual meeting of AOAC INTERNATIONAL in August 2013.

MLT Protocol

As per the MLT protocol (2), the main objective of each participating laboratory was as follows:

- To run the AOAC First Action Method **2012.10** as per the described procedure.
- To perform applicable system suitability tests.
- To analyze 18 selected SPIFAN samples over 2 days in singlicate.
- To send completed data tables for the calibration standards and results, chromatograms, observations, and comments to the Study Director.

Fifteen laboratories representing commercial, industrial, and governmental laboratories in 11 countries agreed to participate in this collaborative study, which was conducted using a blind duplicate design. Each laboratory was requested to first assay two practice samples to ensure that the laboratory analyst understood the entire procedure before proceeding with testing the study samples. All laboratories were asked to provide details including calculations determining the purity of all four standards used and to provide a full set of data, including system suitability checks and chromatograms, to aid the Study Director in evaluating the results and troubleshooting if necessary. Laboratories providing inadequate initial data for the practice samples were provided with troubleshooting assistance before repeating the practice samples. Laboratories providing satisfactory data on the practice samples received a shipment of the collaborative study samples. This test set contained blind duplicates, and each laboratory analyzed each test in singlicate and reported only single-test results.

List of collaborating laboratories:

(*1*) AsureQuality Ltd, Auckland, New Zealand
(*2*) Covance Laboratories, Inc.
(*3*) Perrigo Nutritionals
(*4*) Abbott Laboratories
(*5*) Premium Laboratories, Spain
(*6*) Aerial, France
(*7*) Canadian Food Inspection Agency
(*8*) Nestlé Research Centre, Lausanne, Switzerland
(*9*) Departamento de Desarrollo de Métodos, Technological Laboratory of Uruguay (LATU), Montevideo, Uruguay
(*10*) Eurofins Steins Laboratorium A-S, Denmark
(*11*) Fonterra, New Zealand
(*12*) Mead Johnson Nutrition, Philippines
(*13*) Wyeth Nutrition Ireland
(*14*) Wyeth Nutrition Singapore
(*15*) Wyeth Nutrition Philippines

Method

A slightly modified version of AOAC First Action Method **2012.10** was followed. The main method update was the weighing of the sample diluent and sample aliquot rather than measuring volumetrically. The method, with both variable UV and fluorescence detection, allows for the simultaneous determination of vitamin A palmitate, vitamin A acetate, and total vitamin E in infant, pediatric, and adult nutritional formulas. The procedure utilizes the proteolytic enzyme papain to hydrolyze the hydrophillic protein coating of fat miscelles in milk- or soy-based formulations in an aqueous solution. The hydrophobic contents of the miscelles are then extracted quantitatively into iso-octane in a single extraction and chromatographed by normal-phase HPLC using a Zorbax (Agilent Technologies, Santa Clara, CA) NH_2 analytical column. The analytes are eluted with a gradient and D-α-tocopherol and D-α-tocopherol acetate are quantified using fluorescence detection, excitation/emission, 280/310 nm. Vitamin A palmitate (*cis* and *trans*) and vitamin A acetate (*cis* and *trans*) are quantified using UV detection at 325 nm.

This method meets the applicability statements of the in AOAC *Standard Method Performance Requirements* (SMPR®; 3) **2011.003** (4) for vitamin A and AOAC SMPR **2011.010** (5) for vitamin E as follows:

Vitamin A.—Determination of vitamin A in all forms of infant and adult or pediatric formula [powders, ready-to-feed (RTF) liquids and liquid concentrates]. For the purpose of this SMPR, vitamin A is defined as 13-*cis* and all-*trans* retinol (CAS 68-26-8), retinyl esters [retinyl palmitate (CAS 79-81-2) and retinyl acetate (CAS 127-47-9)].

Vitamin E.—Determination of vitamin E in all forms of infant and adult or pediatric formula (powders, RTF liquids and liquid concentrates), with a focus on D-α-tocopherol (CAS 59-02-9) and all-racemic α-tocopherol (CAS 1406-18-4) and their esters. Methods must be able to report the quantity of α-tocopherol and esters separately.

AOAC Official Method 2012.10
Determination of Vitamins E and A in Infant Formula and Adult Nutritionals

Normal-Phase High-Performance Liquid Chromatography

First Action 2012
Final Action 2014

ISO–AOAC Method

[Applicable to the concurrent quantitative analysis of vitamin E (α-tocopherol and α-tocopherol acetate), vitamin A palmitate, and vitamin A acetate (*cis*- and *trans*-isomers) present in milk- and soy-based infant formula and adult nutritionals and formulas containing hydrolyzed protein. Vitamin A is defined as 13-*cis* and all-*trans* retinol (CAS 68-26-8), retinyl esters [retinyl palmitate (CAS 79-81-2) and retinyl acetate (CAS 127-47-9)]. The determination of vitamin E focuses on α-tocopherol (CAS No. 59-02-9), *all*-racemic α-tocopherol (CAS No. 1406-18-4), and their esters. α-Tocopherol and esters can be reported separately.]

Caution: Correct personal and environmental safety standards shall be used while performing this analytical method. Laboratory personnel handling solvents, acids, and reagents should be knowledgeable of their potential hazards. Consult the Material Safety Data Sheets (MSDS) for information on the hazards and take proper precautions. Transfer solvents and acids inside efficient fume hoods and extractors. Ensure all glassware is free from chipping and hairline cracks.

See Tables **2012.10A** and **B** for results of the method performance studies supporting acceptance of the method.

Table 2012.10A. Method performance requirements: Single-laboratory validation (SLV) and multilaboratory testing (MLT) results summary—Vitamin A[a]

Parameter	Method performance requirements		Retinyl palmitate	Retinyl acetate
Analytical range	7.0–382.6[b]		2–450	2–450
Limit of detection (LOD)	≤2.0[b]		0.099	0.85
Limit of quantitation (LOQ)	≤7.0[b]		0.33	2.83
Repeatability (RSD_r) (SLV)	7–300[b]	≤8%	≤4.03%	≤6.56%
Intermediate precision (RSD_r) (SLV)	7–300[b]	—	≤6.23%	≤10.63%
Recovery (SLV)	90–110% (mean spiked recovery over the range of the assay)		99.13%	96.53%
Reproducibility (RSD_R) (MLT)	10–383[b]	≤16%	6.51–16.25%	11.73–22.61%

[a] Concentrations apply to (*1*) 'ready-to-feed' liquids; (*2*) reconstituted powders (25 g into 200 g water); and (*3*) liquid concentrate diluted 1:1 by weight.

[b] µg/100 g reported separately as *cis*-13 retinol and all-*trans* retinol in reconstituted final product.

A. Principle

This procedure utilizes the proteolytic enzyme papain to hydrolyze the hydrophilic protein coating of fat micelles in milk- or soy-based infant formulations in an aqueous solution. The hydrophobic contents of the micelles are then extracted quantitatively into iso-octane in a single extraction and chromatographed by normal-phase HPLC using a Zorbax® NH2 analytical column. The analytes are eluted with a gradient and α-tocopherol and α-tocopherol acetate quantified using fluorescence detection, excitation/emission, 280/310 nm. Vitamin A palmitate (*cis* and *trans*) and vitamin A acetate (*cis* and *trans*) are quantified using UV detection at 325 nm.

B. Apparatus

Common laboratory glassware and equipment and, in particular, the following:

(**a**) *HPLC system.*—Consisting of pump, autosampler, programmable UV detector operating at 325 nm for vitamin A, and a fluorescence detector (FLD) at an excitation wavelength of 280 nm and an emission wavelength of 310 nm for vitamin E.

(**b**) *HPLC column.*—Analytical normal-phase column, e.g., Zorbax NH2, 5 µm, 150 × 4.6 mm, or equivalent.

(**c**) *Water bath.*—Set at 37 ± 2°C.

(**d**) *Centrifuge.*—With adapters for 50 mL centrifuge tubes, capable of 4000 min^{-1}.

(**e**) *UV-Vis spectrophotometer.*—With 1 cm quartz cells.

(**f**) *Analytical balance.*—Weighing to four decimal places.

(**g**) *Amber HPLC vials.*—2 mL, with plastic caps and polytetrafluoroethylene (PTFE) seals.

(**h**) *Disposable centrifuge tubes.*—50 mL, e.g., Falcon (Fisher, Pittsburgh, PA), or equivalent.

(**i**) *Laboratory mechanical test tube shaker.*

(**j**) *Sonic bath.*

(**k**) *One-mark volumetric flasks.*—50 and 100 mL.

(**l**) *Vacuum filtration apparatus.*—With 0.45 µm nylon membrane.

(**m**) *Laboratory glass bottles.*—250 mL and 1 and 2 L, e.g., Duran (Wertheim/Main, Germany), or equivalent.

(**n**) *Pipettors and tips.*—Gilson P10002, or equivalent.

C. Standards

(**a**) *Vitamin A palmitate reference standard.*—Primary standard, U.S. Pharmacopeial Convention (USP; Rockville, MD, USA), or equivalent. The standard shall contain antioxidant.

Table 2012.10B. Method performance requirements: Single-laboratory validation (SLV) and multilaboratory testing (MLT) results summary—Vitamin E[a]

Parameter	Method performance requirements		α-Tocopherol	α-Tocopherol acetate
Analytical range	0.2–8[b]		0.03–8	0.02–9.4
Limit of detection (LOD)	≤0.1[b]		0.01	0.023
Limit of quantitation (LOQ)	≤0.2[b]		0.035	0.075
Repeatability (RSD_r) (SLV)	0.5–2.0[b]	≤8%	≤4.25%	≤4.39
	4–8[b]	≤6%	≤3.78%	≤3.53%
Intermediate precision (RSD_r) (SLV)	<2.0[b]	—	≤17.31%	≤10.54%
	>2.0[b]	—	≤9.24%	≤8.25%
Recovery (SLV)	90–110% (mean spiked recovery over the range of the assay)		100.60%	102.92%
Reproducibility (RSD_R) (MLT)	0.5–1.0[b]	≤22%	3.84–43.56%	—
	1.0–8.0[b]	≤16%	—	4.15–11.25%
Reproducibility (RSD_R) (MLT) total vitamin E	0.5–1.0[b]	≤22%	3.84–10.78%	
	1.0–8.0[b]	≤16%	≤12.47%	

[a] Concentrations apply to (*1*) 'ready-to-feed' liquids; (*2*) reconstituted powders (25 g into 200 g water); and (*3*) liquid concentrate diluted 1:1 by weight.

[b] mg/100 g α-tocopherol and α-tocopheryl acetate in reconstituted final product.

(**b**) *Vitamin A acetate reference standard*.—Primary standard, USP, or equivalent.

(**c**) *α-Tocopherol acetate reference standard*.—Primary standard, USP, or equivalent.

(**d**) *α-Tocopherol reference standard*.—Primary standard, USP, or equivalent.

D. Chemicals and Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

(**a**) *Methyl-t-butyl ether (also known as tert-butyl methyl ether)*.—HPLC grade.

(**b**) *n-Hexane*.—HPLC grade.

(**c**) *Ethanol*.—HPLC grade.

(**d**) *Methanol*.—HPLC grade.

(**e**) *Iso-octane (2,2,4- trimethylpentane)*.—HPLC grade.

(**f**) *Papain (from Carica papaya)*.—>3 U/mg, Sigma 76220, or equivalent.

(**g**) *Hydroquinone*.—Sigma H90031, or equivalent.

(**h**) *Glacial acetic acid*.—Analytical reagent grade.

(**i**) *Anhydrous sodium acetate*.

(**j**) *Hydrochloric acid*.—36%.

E. Solutions

(**a**) *Dilute hydrochloric acid solution*.—Dilute 100 mL of a hydrochloric acid solution with a mass fraction of 36% to 200 mL with water.

(**b**) *Papain solution, mass concentration ρ = 20 g/L*.—Dissolve 100 mg hydroquinone and 4 g anhydrous sodium acetate in approximately 80 mL water in a 100 mL one-mark volumetric flask. Adjust the pH to 5.0 with dilute hydrochloric acid solution. Add 2 g papain and make up to volume. Prepare fresh prior to use.

(**c**) *Acidified methanol solution*.—Add 20 mL glacial acetic acid to 1 L methanol and mix. Prepare fresh on day of use.

(**d**) *HPLC mobile phase A*.—*n*-Hexane, filtered and degassed for 15 min in an ultrasonic bath.

(**e**) *HPLC mobile phase B*.—Mix 750 mL *n*-hexane with 250 mL methyl-*t*-butyl ether. Add 3 mL methanol, filter, and degas for 15 min in an ultrasonic bath.

F. Calibration Standards

(**a**) *Retinyl palmitate stock standard solution*.—Weigh to the nearest 0.01 mg approximately 70 mg retinyl palmitate into a 50 mL volumetric flask. Dissolve in and dilute to volume with iso-octane.

Table 2012.10C. Pump gradient elution cycle

Time, min	Flow, mL/min	Mobile phase A, %	Mobile phase B, %
0.0	1.5	95	5
3.0	1.5	95	5
12.0	1.5	5	95
14.0	1.5	5	95
15.0	1.5	95	5
20.0	1.5	95	5

(**b**) *Retinyl acetate stock standard solution*.—Weigh to the nearest 0.01 mg approximately 35 mg retinyl acetate into a 50 mL volumetric flask. Dissolve in and dilute to volume with ethanol.

(**c**) *α-Tocopherol acetate stock standard solution*.—Weigh to the nearest 0.01 mg approximately 180 mg α-tocopherol acetate into a 50 mL volumetric flask. Dissolve in and dilute to volume with iso-octane.

(**d**) *α-Tocopherol stock standard solution*.—Weigh to the nearest 0.01 mg approximately 100 mg α-tocopherol into a 50 mL volumetric flask. Dissolve in and dilute to volume with iso-octane.

Note: The above stock standard solutions are stable in a refrigerator at 4–8°C for up to 7 days.

(**e**) *Combined working standard solution 1*.—Transfer by pipet 4 mL retinyl palmitate stock standard solution, 4 mL retinyl acetate stock standard solution, 7 mL α-tocopherol acetate stock standard solution, and 20 mL α-tocopherol stock standard solution into a 50 mL volumetric flask and dilute to volume with iso-octane. Prepare this solution freshly prior to use.

(**f**) *Combined working standard solution 2*.—Transfer by pipet 8 mL combined working standard solution 1 into a 100 mL volumetric flask and dilute to volume with iso-octane. Prepare this solution freshly prior to use.

(**g**) *Calibration standard solutions*.—Into separate 50 mL volumetric flasks, transfer by pipet 0.5, 2, 4, 8, 16, and 32 mL combined working standard solution 2, and dilute to volume with iso-octane. These solutions are used to construct a multipoint calibration curve. Prepare these solutions daily prior to use.

Note: For routine testing and depending on the concentration range of the analytes in the test samples, a 3- or 4-point standard curve can be used, provided the ranges are within the lowest and highest points of the 6-point curve listed above.

If the result of any analyte is outside the calibration range, standard weights and/or dilutions can be adjusted accordingly.

G. Stock Standard Purity Determinations

(**a**) *Spectrometric purity of retinyl palmitate stock solution*.—(*1*) Pipet 1 mL retinyl palmitate stock standard solution into a l00 mL volumetric flask and make up to volume with ethanol.

(*2*) Determine the absorption at 325 nm, zeroed against ethanol in a 1 cm quartz cell. Repeat the reading twice, rinsing the sample cuvet with the solution before each reading.

(*3*) Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_{AP}, of retinyl palmitate using Equation 1:

$$SP_{AP} = \frac{A}{975} \times \frac{50}{m_{st}} \times \frac{100}{1} \times 10 \qquad (1)$$

where A = average absorbance reading, determined above; 975 = extinction coefficient of retinyl palmitate at 325 nm; and m_{st} = mass of the reference standard in mg.

(**b**) *Spectrometric purity of retinyl acetate stock solution*.—(*1*) Pipet 1 mL retinyl acetate stock standard solution into a l00 mL volumetric flask and make up to volume with ethanol.

(*2*) Determine the absorption at 325 nm, zeroed against ethanol, in a 1 cm quartz cell. Repeat the reading twice, rinsing the sample cuvet with the solution before each reading.

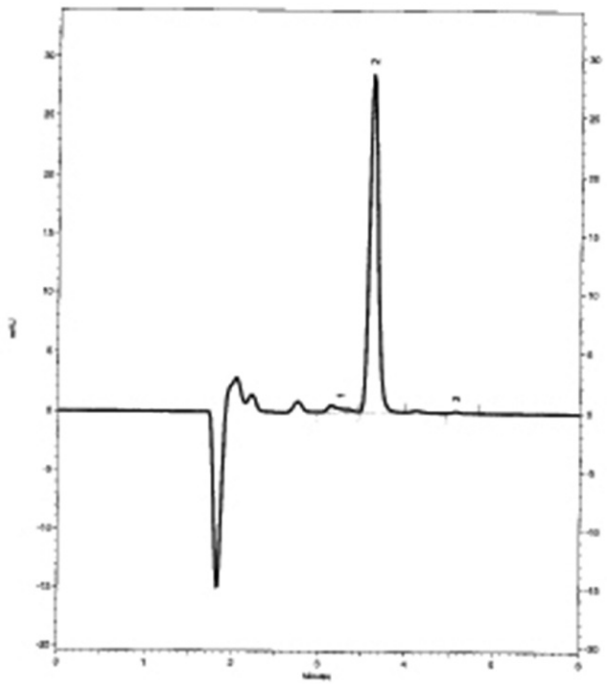

Figure 2012.10A. HPLC chromatogram of vitamin A palmitate calibration standard. Peak 1, ^{13}*cis*-isomer; peak 2, *trans*-isomer.

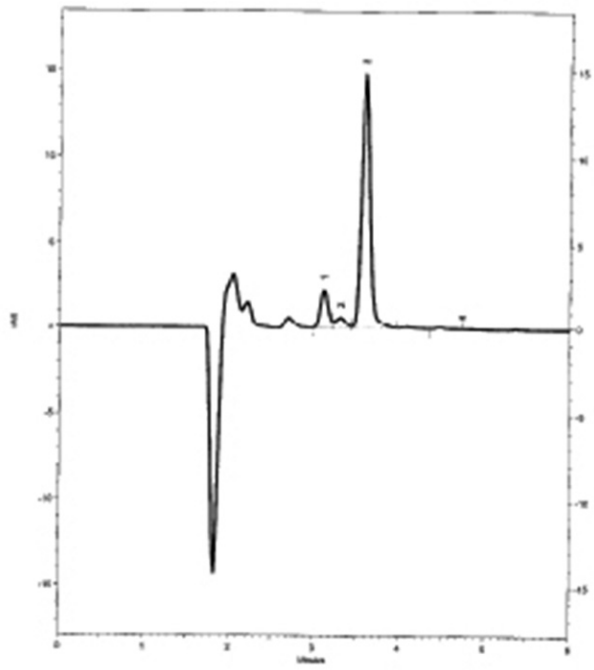

Figure 2012.10B. HPLC chromatogram of vitamin A palmitate test sample. Peak 1, ^{13}*cis*-isomer; peak 2, *cis*-isomer; peak 3, *trans*-isomer.

(*3*) Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_{AA}, of retinyl acetate using Equation 2:

$$SP_{AA} = \frac{A}{1560} \times \frac{50}{m_{st}} \times \frac{100}{1} \times 10 \qquad (2)$$

where A = average absorbance reading, determined above; 1560 = extinction coefficient of retinyl acetate at 325 nm; and m_{st} = mass of the reference standard in mg.

(**c**) *Spectrometric purity of α-tocopherol acetate stock solution.*—(*1*) Pipet 3 mL α-tocopherol acetate stock standard solution into a l00 mL volumetric flask and make up to volume with ethanol.

(*2*) Determine the absorption at 284 nm, zeroed against ethanol, in a 1 cm quartz cell. Repeat the reading twice, rinsing the sample cuvet with the solution before each reading. Calculate the average absorbance reading.

(*3*) Calculate the spectrometric purity as a decimal, SP_{TA}, of α-tocopherol acetate using Equation 3:

$$SP_{TA} = \frac{A}{43.6} \times \frac{50}{m_{st}} \times \frac{100}{3} \times 10 \qquad (3)$$

where A = average absorbance reading, determined above; 43.6 = extinction coefficient of tocopherol acetate at 284 nm; and m_{st} = mass of the reference standard in mg.

(**d**) *Spectrometric purity of α-tocopherol stock solution.*—(*1*) Pipet 3 mL α-tocopherol stock standard solution into a l00 mL volumetric flask and make up to volume with ethanol.

(*2*) Determine the absorption at 292 nm, zeroed against ethanol in a 1 cm quartz cell. Repeat the reading twice, rinsing the sample cuvet with the solution before each reading.

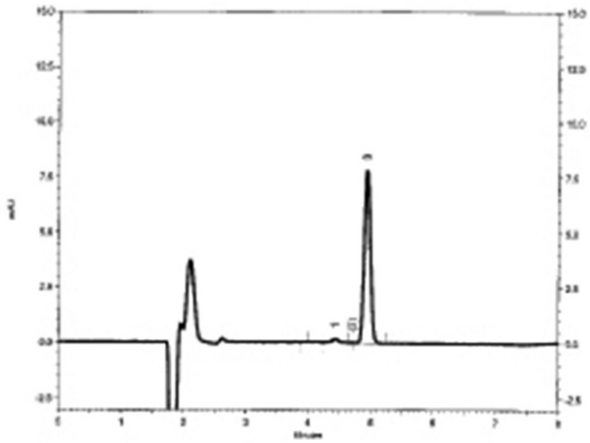

Figure 2012.10C. HPLC chromatogram of vitamin A acetate calibration standard. Peak 1, ^{13}*cis*-isomer; peak 3, *trans*-isomer.

(*3*) Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_T, of α-tocopherol using Equation 4:

$$SP_T = \frac{A}{75.8} \times \frac{50}{m_{st}} \times \frac{100}{3} \times 10 \qquad (4)$$

where A = average absorbance reading, determined above; 75.8 = extinction coefficient of tocopherol at 292 nm; and m_{st} = mass of the reference standard in mg.

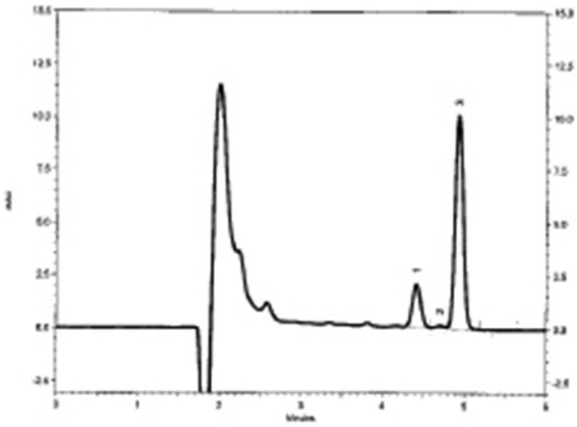

Figure 2012.10D. HPLC chromatogram of vitamin A acetate test sample. Peak 1, ^{13}*cis*-isomer; peak 2, *cis*-isomer; peak 3, *trans*-isomer.

(**e**) *Chromatographic purity of stock standard solutions*.—Prepare each stock standard solution separately as follows:

(*1*) Into four separate 100 mL volumetric flasks transfer by pipet 1 mL of each of the stock standard solutions, retinyl palmitate, retinyl acetate, α-tocopherol acetate, and α-tocopherol. Label each flask with the individual analyte names.

(*2*) Mix and dilute each to volume with iso-octane.

(*3*) Into four separate labeled 2 mL autosampler vials transfer by autopipettor 60 µL retinyl palmitate solution, 30 µL retinyl acetate solution, 100 µL α-tocopherol acetate solution, and 400 µL α-tocopherol. Fill vial with iso-octane to approximately 2 mL.

(*4*) Vortex briefly and inject into the LC system according to the method parameters described in **G**. Analyze vitamin A palmitate and vitamin A acetate by UV at 325 nm. For α-tocopherol acetate, analyze by UV at 284 nm and for α-tocopherol, analyze at 292 nm.

(*5*) Calculate the chromatographic purity (CP) as a decimal for each peak of interest after integration of all the peaks appearing on each chromatogram, using Equation 5:

$$CP = A/100 \qquad (5)$$

where CP = area of peak of interest/total peak area excluding solvent.

(**f**) *Calculation of the concentrations of working standard solutions*.—Calculate the concentration, ρ_w, of each vitamin in the working standard solutions from the stock solution concentration using the appropriate dilution factor as shown in Equations 6 to 9 in µg/mL for retinyl palmitate (RP) and retinyl acetate (RA) and mg/mL for α-tocopherol (T) and α-tocopherol acetate (TA).

$$\rho_{wRP} = SP_{RP} \times CP_{RP} \times \frac{m_{st}}{50} \times \frac{4}{50} \times \frac{8}{100} \times \frac{V_a}{50} \times 1000 \qquad (6)$$

$$\rho_{WRA} = SP_{RA} \times CP_{RA} \times \frac{m_{st}}{50} \times \frac{4}{50} \times \frac{8}{100} \times \frac{V_a}{50} \times 1000 \qquad (7)$$

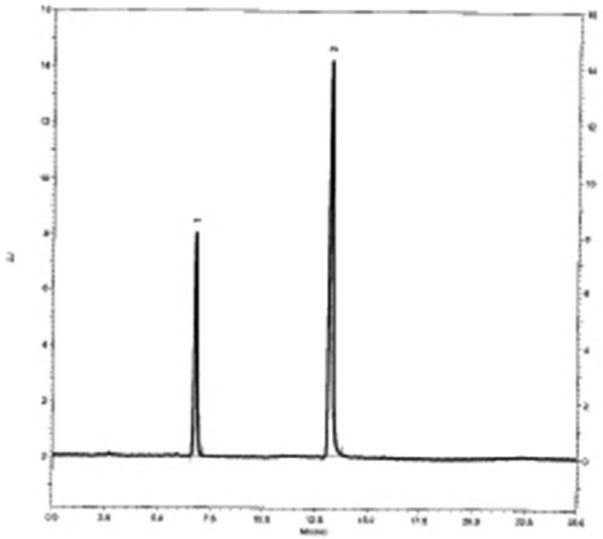

Figure 2012.10E. HPLC chromatogram of α-tocopherol acetate and α-tocopherol calibration standard. Peak 1, α-tocopherol acetate; peak 2, α-tocopherol.

$$\rho_{wT} = SP_T \times CPT \times \frac{m_{st}}{50} \times \frac{7}{50} \times \frac{8}{100} \times \frac{V_a}{50} \qquad (8)$$

$$\rho_{WTA} = SP_{TA} \times SP_{TA} \times \frac{m_{st}}{50} \times \frac{20}{50} \times \frac{8}{100} \times \frac{V_a}{50} \qquad (9)$$

where V_a = 0.5, 2, 4, 8, 16, and 32 mL, respectively, for the calibration levels; m_{st} = mass of the reference standard in mg; SP = UV spectrometric purity as a decimal; CP = chromatographic purity as a decimal; and 1000 = conversion factor from mg/mL to µg/mL.

H. Sample Preparation

(**a**) For dry blended/nonhomogenous powder samples, transfer 25 g, accurately weighed, to a 250 mL bottle. Dissolve using warm water (about 40–45°C), cool, and make up to 200 g with water. Note the final weight (m_2). Transfer 5.0 g (m_3) reconstituted sample to a screw-top centrifuge tube. Calculate the sample mass (powder equivalent), m_s, using Equation 10:

$$m_s = \frac{(m_1 \times m_3)}{m_2} \qquad (10)$$

(**b**) For wet blended homogenous powder samples, transfer 0.5000–0.5500 g, accurately weighed, directly to a screw-top 50 mL centrifuge tube. Add 5 mL warm water of approximately 40°C and shake to dissolve.

(**c**) For ready-to-feed samples or concentrated liquid products, transfer 5.0 g (m_3) thoroughly homogenized sample directly to a screw-top 50 mL centrifuge tube.

(**d**) To the above weighed solutions, add 5 mL papain solution. Mix to disperse each sample, cap, and place the tubes in a 37±2°C water bath for 20–25 min.

(**e**) Remove the samples from the water bath and cool. Place in a freezer for about 5 min or refrigerate for about 20 min.

(**f**) Add 20 mL acidified methanol to each sample tube and shake tubes for 10 min, preferably with a mechanical shaker.

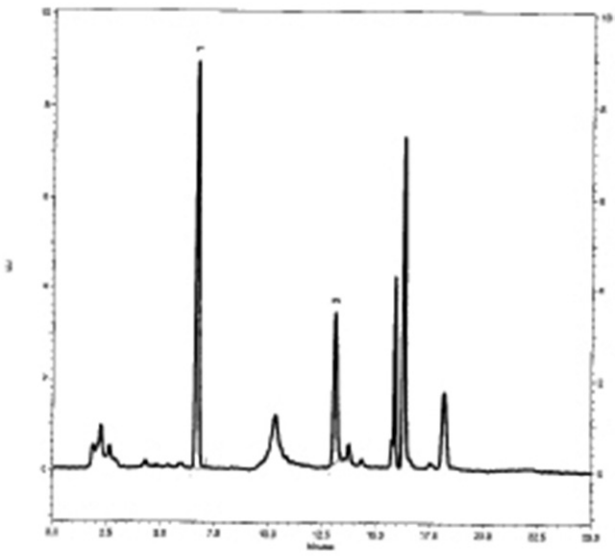

Figure 2012.10F. HPLC chromatogram of α-tocopherol acetate and α-tocopherol sample chromatogram. Peak 1, α-tocopherol acetate; peak 2, α-tocopherol.

(**g**) Volumetrically pipet 10.0 mL iso-octane to each sample tube. Close tightly to avoid leakage and shake tubes for 10 min, preferably with a mechanical shaker.

(**h**) Centrifuge for 10 min at 4000 min^{-1} to obtain a clear iso-octane layer.

(**i**) Transfer an aliquot of the clear iso-octane layer into amber vials for HPLC analysis.

I. HPLC Analysis

Separation and quantification have proven to be satisfactory if the following experimental conditions are followed:

Column.—Zorbax NH2 (5 µm, 150 × 4.6 mm).

Mobile phase A.—*n*-Hexane.

Mobile phase B.—Mixture of 750 mL *n*-hexane, 250 mL methyl-*t*-butyl ether, and 3 mL methanol.

Flow rate.—1.5 mL/min.

Injection volume.—50 µL.

Column oven.—40 ± 2°C.

Run time.—20 min.

Detector settings.—Set the photodiode array (PDA)/UV detector at 325 nm for vitamin A palmitate and vitamin A acetate. Set the fluorescence detector at excitation wavelength of 280 nm and emission wavelength of 310 nm for α-tocopherol acetate and α-tocopherol.

Pump gradient elution cycle.—*See* Table **2012.10C**.

Examples for typical chromatograms are given in Figures **2012.10A–F**.

Note: The gradient given can be altered as required to maximize the analytical separation and avoid interferences.

J. System Suitability

The following system suitability and standard checks should be met when running this method.

(**a**) The coefficient of determination, R^2, of each calibration curve should be ≥0.995.

(**b**) The resolution between *cis* and *trans* vitamin A palmitate and between *cis* and *trans* vitamin A acetate in the reference standard should be ≥1.5.

K. Calculations

Calculate the concentration, *w*, of the sample in µg/100 g for retinyl palmitate or retinyl acetate and mg/100 g for α-tocopherol or α-tocopherol acetate (powder or liquid).

$$w = \frac{(A_s - I)}{S} \times V_{iso} \times \frac{100}{m_s} \qquad (11)$$

where A = peak area or height of retinyl palmitate or retinyl acetate or α-tocopherol or α-tocopherol acetate in the test sample solution; I = intercept of the calibration curve; S = slope of the calibration curve; V_{iso} = volume of iso-octane used (here, V_{iso} = 10 mL); 100 = factor to convert in 100 g basis; and m_s = sample mass (for liquid samples) or powder equivalent in g (powder samples).

For the purposes of this method there is no differentiation of the varying contributions of cis- and transisomers to the total vitamin A palmitate/acetate activity.

For vitamin A peak integration, sum the area of the 13-cis and all trans isomers of vitamin A palmitate/acetate and calculate against the trans isomer.

To convert vitamin A results to retinol using stoichiometric calculations in accordance with Equation 12:

Vitamin A as retinol (µg/100 g) =
(retinyl palmitate in µg/100 g × 0.55)
+ (retinyl acetate in µg/100 g × 0.87)

Convert vitamin E results to α-tocopherol using stoichiometric calculations:

— 1 mg of α-tocopheryl acetate is equal to 1,10 α-tocopherol, and — 1 mg = 1,10 DL α-tocopherol (synthetic vitamin E; all racemic α-tocopherol).

Results and Discussion

System Suitability and Linearity

All system suitability checks performed during this collaborative study met the following acceptance criteria:

(a) The resolution between the *cis* and *trans* forms of vitamin A palmitate and *cis* and *trans* forms of vitamin A acetate were baseline separated.

(b) Standard injection precision was <2.0%.

(c) The coefficient of determination R^2 of all standard curves generated during the study exceeded the minimum requirement of ≥0.995.

Practice Samples

Two practice samples (both from milk based formula, one fortified with Vitamin A palmitate and one fortified with Vitamin A

Acetate and both fortified with α-tocopherol acetate) were used by the participating laboratories so that the laboratories could become familiar with the analysis procedure. The results were submitted to the Study Director for approval prior to commencing the collaborative study. Results within a range of expected levels indicated that the laboratory was capable of successfully running the analysis. The same practice samples were used as QC samples during the analysis of the study samples.

Most of the laboratories submitted practice sample results that met the acceptance criteria. A couple of laboratories submitted results just outside the acceptance limits but were accepted to participate in the study as they met the acceptance limits for at least three of the four analytes.

Milk-Based Products

For retinyl palmitate, RSD_r was calculated in a range of 1.06–4.72% and RSD_R in a range of 6.51–10.52%.

For retinyl acetate, RSD_r was 16.60% and RSD_R was 22.61%.

For α-tocopherol acetate, RSD_r was calculated in a range of 0.60–3.84% and RSD_R in a range of 4.15–8.67%.

For α-tocopherol, RSD_r was calculated in a range of 1.57–5.78% and RSD_R in a range of 5.68–12.47%.

For total vitamin E, RSD_r as tocopherol equivalents (TEs) was calculated in a range of 0.81–3.74% and RSD_R in a range of 3.84–7.17%.

Note: One milligram of α-tocopherol acetate is equal to 0.671 mg TEs. One milligram of α-tocopherol is equal to 1 mg TEs.

Low Fat Product

For total vitamin A palmitate, RSD_r was 15.78% and RSD_R was 21.73%.

For α-tocopherol acetate, RSD_r was 2.11% and RSD_R was 8.50%.

For α-tocopherol, RSD_r was 8.90% and RSD_R was 43.56%.

For total vitamin E, RSD_r as TEs was 2.71% and RSD_R was 10.78%.

The concentration of α-tocopherol was relatively low in the product: 0.14 mg/100 g ready to feed (RTF).

Hydrolyzed Protein and Elemental Products

For retinyl acetate, the partially hydrolyzed soy-based product gave an RSD_r of 2.30% and an RSD_R of 11.93%.

For α-tocopherol acetate, RSD_r was calculated as 3.65% and RSD_R was 11.25%.

For α-tocopherol, RSD_r was calculated as 1.67% and RSD_R was 11.94%.

For total vitamin E as TEs, RSD_r was calculated as 5.46% and RSD_R was 10.15%.

For retinyl palmitate, the elemental product gave an RSD_r of 15.13% and an RSD_R of 16.25%.

For α-tocopherol acetate, RSD_r was calculated as 3.38% and RSD_R was 6.66%.

For α-tocopherol, RSD_r was calculated as 15.48% and RSD_R was 17.44%.

For total vitamin E as TEs, RSD_r was calculated as 4.90% and RSD_R was 5.68%.

Soy Product

For retinyl palmitate, RSD_r was 6.84% and RSD_R was 9.66%.

For α-tocopherol acetate, RSD_r was calculated as 1.67% and RSD_R was 6.47%.

For α-tocopherol, RSD_r was calculated as 7.89% and RSD_R was 8.74%.

For total vitamin E as TEs, RSD_r was calculated as 2.07% and RSD_R was 4.22%.

For two blind duplicate samples of the milk-based hydrolyzed protein formula, where the repeatability was observed as being very high (up to 50% RSDs), repeat analysis was performed by a selection of laboratories. All initial results were confirmed. Repeatability obtained at the SLV stage (duplicates over 6 separate days) was very good for this matrix, 5.4 and 5.78% for *cis* and *trans* retinyl palmitate and 5.5 and 10.2% for α-tocopherol acetate and α-tocopherol. Results obtained for this sample were deemed invalid due to the material being expired at time of testing following an investigation and confirmation that other study directors encountered similar difficulties and following a discussion at the September 2014 ERP meeting. The results for this sample have been removed from the AOAC Final Action Method document.

AOAC INTERNATIONAL *Interlaboratory Study Workbook Revision 2.1* (6) was used to perform the statistical evaluation of the data. Outliers were detected and reviewed prior to exclusion from the data set. Where possible, detected outliers were reviewed by the participating laboratories for possible transcription or calculation errors. Across the four anaytes, 13-cis and all-trans Vitamin A, and α-tocopherol acetate and α-tocopherol, laboratories 4–8 and 10–12 had no statistical outliers. Laboratory 13 had one outlier, laboratories 1 and 15 had 2 outliers, laboratory 2 had 3 outliers, laboratory 3 had 4 outliers, laboratories 9 and 14 had 14 outliers. The statistical summary (Tables 1-20) lists the outliers and the reasons for removal (Cochran test outlier or Single Grubbs test outlier), as well as Cochran test or single Grubbs test outliers.

An invitation was sent to all participating laboratories to comment on the performance of the method in their laboratories. In general, the comments were very positive. Laboratory 3 indicated that sample preparation was easy. According to the laboratory, it is not necessary to prepare the working standard (6 point curve) fresh daily, as the reagent used to prepare the stock and the working standards is the same. Laboratory 3 also indicated that the papain solution should be sonicated to ensure it is fully dissolved. Laboratory 4 changed the dilution of the standards to be within the working range of the spectrophotometric measurements and HPLC calibration curves. It used an injection volume of 35 µL instead of 50. An injection volume between 20 and 100 µL can be used with the method, depending on sensitivity. The laboratory indicated that 35 µL presented areas (or heights) to give optimum quantification and optimization. Laboratory 5 commented that the sample preparation procedure is

easy, quick (high throughput), and very much fit/suitable in a routine testing environment. Laboratory 6 commented that the calibration curves for vitamin A palmitate, vitamin A acetate, and α-tocopherol were too large, so the sample weight was adjusted to fit the curves. It had no problems with sample preparation, finding it easy to do. It has an advantage over the saponification method as it measures the esters separately. Laboratory 7 found that the method offered very good precision in terms of repeatability and reproducibility. It also noted that the standards and reagents/solutions were easy to prepare and that the sample preparation was simple and stable. It indicated that the method only takes 3 h to prepare a minimum of 15 samples; even less time is needed if not reconstituting, and still allowing determination of retinyl palmitate, retinyl acetate, α-tocopherol acetate, and α-tocopherol. Saponification procedures cannot separate α-tocopherol acetate and α-tocopherol. Laboratory 10 found that the method was very straight-forward and encountered no issues with the protocol or samples. Laboratory 11 found that the method performed well in its laboratory for most of the SPIFAN matrixes, however, it experienced a few difficulties with the sample preparation and would prefer to use vortex mixers or stir plates to a mechanical shaker. It found that with some matrixes, the samples were difficult to mix well with their model of mechanical shaker.

Conclusions

The purpose of this standard is to provide a simple, accurate analytical method for the analysis of total vitamin A and total vitamin E in infant formula and adult nutritional products, while also meeting the applicability statements and complying with the performance acceptance criteria outlined in the SPIFAN SMPRs (4, 5).

Cis and *trans* isomers of vitamin A (palmitate and acetate) and α-tocopherol acetate and α-tocopherol can be separately quantified by UV and fluorescence detection. Compared with other methods for the analysis of these fat-soluble vitamins, this method is considered more rapid and efficient, providing good performance and ease of implementation for routine use in a QC environment.

The collaborative study included 15 laboratories. Some had experience in using this method while others had no previous experience. Low repeatability for the majority of matrix types across the laboratories indicates that relatively little experience is required to precisely and efficiently run this method. The method was applied to a variety of different infant formulas and adult nutritional product types and demonstrated acceptable reproducibility precision across the analytes.

Table 1. Precision data for vitamin A retinyl acetate (a and b) and retinyl palmitate (c–h)

Sample	Adult nutritional powder milk protein-based	Infant formula powder partially hydrolyzed soy-based	SRM 1849a[a]	Adult nutritional powder low fat	Infant formula			Infant elemental powder
					Powder soy-based	Powder milk-based	RTF milk-based[b]	
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
No. of laboratories	15	15	15	15	15	15	15	15
No. of laboratories retained after eliminating outliers	15	12	15	15	15	14	14	15
No. of outliers (laboratories)	0	3	0	0	0	1	1	0
No. of accepted results	30	24[c,d]	30	30	30	28[c]	28[c]	30
Mean value $\bar{x}$, µg/100 g RTF	46.34	67.39	6.49	47.55	62.56	66.58	57.34	48.35
Repeatability SD s_r, µg/100 g RTF	7.69	1.55	0.21	7.50	4.28	0.75	0.61	7.31
Reproducibility SD s_R, µg/100 g RTF	10.48	8.04	0.52	10.33	6.04	4.33	4.13	7.86
RSD_r, %	16.60	2.30	3.26	15.78	6.84	1.13	1.06	15.13
RSD_R, %	22.61	11.93	8.02	21.73	9.66	6.51	7.20	16.25
Repeatability limit r ($r = 2.8 \times s_r$), µg/100 g RTF	21.54	4.34	0.59	21.01	11.98	2.10	1.70	20.48
Reproducibility limit R ($R = 2.8 \times s_R$), µg/100 g RTF	29.34	22.52	1.46	28.92	16.91	12.13	11.56	21.99
HorRat value	1.25	0.69	0.33	1.20	0.56	0.38	0.41	0.91

[a] Milligrams per kilogram powder (National Institute of Standards and Technology, Gaithersburg, MD).

[b] RTF = Ready-to-feed.

[c] Cochran test outlier.

[d] Grubbs test outlier.

Table 2. MLT precision data for vitamin E D-α-tocopherol

Sample	Adult nutritional powder milk protein-based	Infant formula powder partially hydrolyzed soy-based	SRM 1849a[a]	Adult nutritional powder low fat	Infant formula			Infant elemental powder
					Powder soy-based	Powder milk-based	RTF milk-based[b]	
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
No. of laboratories	15	15	15	15	15	15	15	15
No. of laboratories retained after eliminating outliers	14	11	14	14	14	13	14	14
No. of outliers (laboratories)	1	4	1	1	1	0	1	1
No. of accepted results	28[c]	22[c,d]	28[c]	28[d]	28[c,d]	26[c,d]	28[c]	28[c]
Mean value $\bar{x}$, mg/100 g RTF	0.58	0.48	36.38	0.14	0.46	0.49	0.45	0.41
Repeatability SD s_r, mg/100 g RTF	0.023	0.008	2.103	0.012	0.036	0.008	0.012	0.064
Reproducibility SD s_R, mg/100 g RTF	0.052	0.057	4.539	0.061	0.040	0.279	0.035	0.072
RSD_r, %	3.99	1.67	5.78	8.90	7.89	1.57	2.59	15.48
RSD_R, %	9.10	11.94	12.47	43.56	8.74	5.68	7.73	17.44
Repeatability limit r ($r = 2.8 \times s_r$), mg/100 g RTF	0.064	0.022	5.889	0.035	0.101	0.022	0.033	0.178
Reproducibility limit R ($R = 2.8 \times s_R$), mg/100 g RTF	0.147	0.159	12.71	0.170	0.112	0.078	0.098	0.201
HorRat value	0.930	0.920	1.870	2.180	0.680	0.440	0.600	1.340

[a] Milligrams per kilogram powder.

[b] RTF = Ready-to-feed.

[c] Grubbs test outlier.

[d] Cochran test outlier.

Table 3. MLT precision data for vitamin E D-α-tocopherol acetate

Sample	Adult nutritional powder milk protein-based	Infant formula powder partially hydrolyzed soy-based	SRM 1849a[a]	Adult nutritional powder low fat	Infant formula			Infant elemental powder
					Powder soy-based	Powder milk-based	RTF milk-based[b]	
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
No. of laboratories	15	15	15	15	15	15	15	15
No. of laboratories retained after eliminating outliers	14	15	14	13	14	13	13	15
No. of outliers (laboratories)	1	0	0	0	0	2	2	0
No. of accepted results	28[c]	30	28[d]	26[c,d]	28[d]	26[c,d]	26[c,d]	30
Mean value $\bar{x}$, mg/100 g RTF	12.73	1.80	172.89	1.84	1.30	1.44	1.56	1.79
Repeatability SD s_r, mg/100 g RTF	0.489	0.066	3.374	0.388	0.022	0.021	0.009	0.061
Reproducibility SD s_R, mg/100 g RTF	0.926	0.203	14.991	0.156	0.084	0.067	0.065	0.119
RSD_r, %	3.84	3.65	1.95	2.11	1.67	1.43	0.60	3.38
RSD_R, %	7.28	11.25	8.67	8.50	6.47	4.62	4.15	6.66
Repeatability limit r ($r = 2.8 \times s_r$), mg/100 g RTF	1.369	0.184	9.447	0.109	0.061	0.058	0.026	0.170
Reproducibility limit R ($R = 2.8 \times s_R$), mg/100 g RTF	2.594	0.567	41.976	0.437	0.236	0.187	0.181	0.334
HorRat value	0.330	1.070	0.580	0.810	0.580	0.420	0.380	0.630

[a] Milligrams per kilogram powder.

[b] RTF = Ready-to-feed.

[c] Grubbs test outlier.

[d] Cochran test outlier.

Table 4. MLT precision data for total vitamin E as TE

Sample	Adult nutritional powder milk protein-based	Infant formula powder partially hydrolyzed soy-based	SRM 1849a[a]	Adult nutritional powder low fat	Infant formula			Infant elemental powder
					Powder soy-based	Powder milk-based	RTF milk-based[b]	
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
No. of laboratories	15	15	15	15	15	15	15	15
No. of laboratories retained after eliminating outliers	14	15	12	14	14	14	14	15
No. of outliers (laboratories)	1	0	3	1	1	1	1	0
No. of accepted results	28[c]	30	24[c,d]	28[d]	28[d]	28[d]	28[d]	30
Mean value $\bar{x}$, mg/100 g RTF	13.30	2.27	209.24	1.95	1.79	1.94	2.01	2.22
Repeatability SD s_r, mg/100 g RTF	0.50	0.12	4.06	0.05	0.04	0.03	0.02	0.10
Reproducibility SD s_R, mg/100 g RTF	0.95	0.23	8.18	0.21	0.08	0.08	0.08	0.12
RSD_r, %	3.74	5.46	1.94	2.71	2.07	1.32	0.81	4.70
RSD_R, %	7.17	10.15	3.91	10.78	4.22	4.13	3.84	5.55
Repeatability limit r ($r = 2.8 \times s_r$), mg/100 g RTF	10.46	15.28	5.44	7.58	5.79	3.70	2.27	13.17
Reproducibility limit R ($R = 2.8 \times s_R$), mg/100 g RTF	20.09	28.43	10.94	30.19	11.81	11.57	10.74	15.53
HorRat value	0.93	1.00	0.27	1.04	0.68	0.40	0.37	0.55

[a] Milligrams per kilogram powder.

[b] RTF = Ready-to-feed.

[c] Grubbs test outlier.

[d] Cochran test outlier.

Table 5. Collaborative data for adult nutritional powder milk protein-based

Laboratory No.	*Cis*		*Trans*		Total vitamin A as RE (retinol equivalents)	
	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	2.56	2.70	43.47	46.68	46.03	49.38
Laboratory 2	2.55	2.18	47.96	37.63	50.51	39.80
Laboratory 3	3.06	2.32	52.54	39.76	55.60	42.08
Laboratory 4	3.39	3.20	60.76	50.04	64.15	53.24
Laboratory 5	2.50	2.63	40.60	43.45	43.10	46.08
Laboratory 6	2.28	2.01	38.06	38.98	40.34	40.99
Laboratory 7	4.07	2.62	62.54	43.91	66.61	46.53
Laboratory 8	3.18	2.93	38.38	49.62	41.56	52.55
Laboratory 9	3.13	12.10	39.57	35.78	42.70	47.88
Laboratory 10	2.33	2.18	44.52	35.77	46.85	37.96
Laboratory 11	1.69	2.45	31.58	45.08	33.28	47.53
Laboratory 12	2.36	1.88	38.81	31.05	41.17	32.93
Laboratory 13	1.62	3.12	29.09	47.49	30.70	50.61
Laboratory 14	8.04	4.31	58.14	64.81	66.18	69.12
Laboratory 15	2.05	1.84	27.46	33.38	29.51	35.22

Table 6. Collaborative data for infant formula powder partially hydrolyzed soy-based

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	15.71	15.71	47.93	51.11	63.64	66.83
Laboratory 2	16.45[a]	6.64[a]	58.37[a]	18.22[a]	74.82[a]	24.86[a]
Laboratory 3	16.00[a]	7.83[a]	51.48[a]	20.63[a]	67.48[a]	28.45[a]
Laboratory 4	19.53	19.22	61.29	58.14	80.83	77.36
Laboratory 5	17.89	17.88	50.59	50.84	68.48	68.71
Laboratory 6	15.22	14.09	51.87	48.88	67.09	62.97
Laboratory 7	20.56	19.54	62.14	64.28	82.70	83.82
Laboratory 8	15.83	16.67	52.36	52.19	68.18	68.86
Laboratory 9	14.34	15.23	46.86	47.93	61.20	63.16
Laboratory 10	17.11	17.12	54.69	54.70	71.79	71.82
Laboratory 11	14.76	15.44	49.92	49.87	64.67	65.30
Laboratory 12	14.82	15.90	49.03	46.91	63.85	62.81
Laboratory 13	14.28	14.56	51.25	47.75	65.53	62.31
Laboratory 14	25.67[a]	27.03[a]	93.72[a]	84.35[a]	119.39[a]	111.38[a]
Laboratory 15	12.66	12.66	39.58	40.53	52.24	53.20

[a] Cochran and/or Grubbs outliers.

Table 7. Collaborative data for SRM 1849a

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	1.31	1.31	4.76	4.73	6.08	6.03
Laboratory 2	1.53	1.55	5.41	5.40	6.94	6.95
Laboratory 3	1.43	1.37	4.96	4.83	6.39	6.19
Laboratory 4	1.52	1.50	5.19	5.20	6.71	6.69
Laboratory 5	1.41	1.51	4.83	5.09	6.23	6.60
Laboratory 6	1.26	1.21	4.41	4.49	5.68	5.69
Laboratory 7	1.52	1.52	5.20	5.12	6.72	6.65
Laboratory 8	1.44	1.48	5.21	5.31	6.65	6.80
Laboratory 9	1.45	1.22	5.03	4.32	6.48	5.54
Laboratory 10	1.52	1.72	5.43	5.66	6.95	7.37
Laboratory 11	1.46	1.58	5.10	5.23	6.56	6.81
Laboratory 12	1.36	1.40	4.78	4.80	6.14	6.19
Laboratory 13	1.65	1.63	5.91	5.88	7.56	7.51
Laboratory 14	1.42	1.38	5.05	4.98	6.47	6.36
Laboratory 15	1.28	1.26	4.62	4.66	5.90	5.92

Table 8. Collaborative data for adult nutritional powder low fat

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	6.67	10.34	29.25	44.20	35.92	54.54
Laboratory 2	8.34	10.75	38.91	50.02	47.25	60.77
Laboratory 3	8.72	7.81	40.21	35.86	48.93	43.67
Laboratory 4	8.68	11.19	38.52	48.00	47.20	59.19
Laboratory 5	10.82	7.87	46.60	35.12	57.42	42.98
Laboratory 6	9.09	9.39	41.89	42.12	50.98	51.51
Laboratory 7	8.15	8.22	37.78	39.49	45.93	47.71
Laboratory 8	8.20	10.21	39.05	47.04	47.26	57.25
Laboratory 9	9.32	5.39	41.30	22.57	50.62	27.96
Laboratory 10	11.07	10.11	48.76	47.89	59.83	57.99
Laboratory 11	9.25	11.33	38.73	47.02	47.98	58.35
Laboratory 12	9.33	8.34	42.29	39.42	51.62	47.76
Laboratory 13	3.84	4.66	22.17	25.92	26.01	30.58
Laboratory 14	9.74	9.53	46.56	43.99	56.30	53.53
Laboratory 15	4.85	5.38	23.41	25.81	28.26	31.19

Table 9. Collaborative data for infant elemental powder

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	3.53	3.09	46.48	37.57	50.01	40.66
Laboratory 2	3.16	3.13	37.82	37.10	40.98	40.23
Laboratory 3	4.70	3.60	52.84	44.01	57.53	47.61
Laboratory 4	3.02	3.43	42.54	39.20	45.56	42.63
Laboratory 5	4.05	5.21	40.44	51.70	44.49	56.90
Laboratory 6	3.32	2.74	43.05	35.83	46.37	38.57
Laboratory 7	3.53	5.55	43.61	57.42	47.14	62.98
Laboratory 8	3.73	3.89	43.75	46.90	47.48	50.79
Laboratory 9	5.30	4.47	51.09	44.48	56.38	48.95
Laboratory 10	4.47	7.88	41.01	61.67	45.48	69.55
Laboratory 11	3.70	2.85	34.23	35.44	37.94	38.29
Laboratory 12	2.45	3.78	45.00	52.41	47.45	56.18
Laboratory 13	2.46	2.27	40.45	37.83	42.91	40.10
Laboratory 14	5.24	3.66	55.56	43.28	60.80	46.94
Laboratory 15	3.73	4.19	43.66	47.89	47.39	52.08

Table 10. Collaborative data for infant formula powder milk-based

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	11.94	11.92	54.49	54.06	66.43	65.98
Laboratory 2	12.49	12.52	56.41	55.72	68.90	68.23
Laboratory 3	11.67	11.68	53.73	54.04	65.40	65.72
Laboratory 4	10.96	10.86	56.69	56.33	67.65	67.19
Laboratory 5	12.93	12.92	56.22	56.48	69.15	69.40
Laboratory 6	10.19	10.37	51.70	51.13	61.89	61.50
Laboratory 7	12.76	12.78	54.63	56.40	67.39	69.18
Laboratory 8	11.70	11.81	56.37	57.88	68.07	69.69
Laboratory 9	12.12[a]	9.20[a]	52.39[a]	39.08[a]	64.50	48.27
Laboratory 10	14.56	14.29	62.41	61.46	76.96	75.75
Laboratory 11	12.29	12.30	56.16	54.91	68.45	67.21
Laboratory 12	10.60	10.58	54.36	53.18	64.96	63.75
Laboratory 13	10.05	9.73	50.44	50.59	60.49	60.32
Laboratory 14	11.96	12.49	55.00	56.53	66.96	69.02
Laboratory 15	10.43	10.21	48.83	49.11	59.26	59.31

[a] Cochran and/or Grubbs outliers.

Table 11. Collaborative data for infant formula RTF milk-based

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	16.23	16.33	37.54	36.91	53.77	53.24
Laboratory 2	18.06	17.89	42.26	42.34	60.31	60.23
Laboratory 3	17.40	17.30	40.96	40.25	58.36	57.55
Laboratory 4	17.17	17.36	40.45	40.75	57.61	58.11
Laboratory 5	18.17	17.96	41.56	41.42	59.73	59.38
Laboratory 6	14.95	15.48	37.85	37.70	52.79	53.18
Laboratory 7	18.19	18.31	40.64	41.50	58.83	59.81
Laboratory 8	17.12	17.03	41.54	41.92	58.66	58.94
Laboratory 9	16.67	14.52	38.35[a]	33.44[a]	55.02	47.96
Laboratory 10	22.42	20.57	45.97	45.55	68.39	66.12
Laboratory 11	16.90	17.12	40.63	40.69	57.53	57.81
Laboratory 12	15.61	15.82	38.11	38.26	53.73	54.08
Laboratory 13	15.29	15.52	39.92	39.66	55.21	55.18
Laboratory 14	17.72	17.16	41.13	40.39	58.85	57.55
Laboratory 15	13.80	14.25	36.05	36.49	49.85	50.74

[a] Cochran and/or Grubbs outliers.

Table 12. Collaborative data for infant formula powder soy-based

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	14.05	14.19	48.05	47.17	62.10	61.36
Laboratory 2	14.28	14.16	50.80	49.35	65.08	63.51
Laboratory 3	13.08	13.32	47.81	49.78	60.89	63.10
Laboratory 4	15.58	14.63	53.36	41.23	68.94	55.87
Laboratory 5	14.91	14.80	52.28	52.76	67.19	67.57
Laboratory 6	12.36	11.77	47.09	47.36	59.45	59.13
Laboratory 7	15.21	14.34	53.56	52.78	68.77	67.12
Laboratory 8	13.47	15.64	48.68	42.63	62.15	58.27
Laboratory 9	9.56	13.40	33.79	48.50	43.35	61.90
Laboratory 10	17.19	16.56	54.44	57.11	71.64	73.67
Laboratory 11	14.35	14.95	51.41	51.58	65.76	66.53
Laboratory 12	12.80	13.15	49.71	47.96	62.51	61.11
Laboratory 13	11.26	10.66	44.58	44.74	55.84	55.40
Laboratory 14	14.55	14.00	52.77	52.72	67.31	66.72
Laboratory 15	11.64	12.11	45.00	45.77	56.64	57.88

Table 13. Collaborative data for adult nutritional powder milk protein-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	12.62	13.11	0.55	0.50	8.88	9.16
Laboratory 2	12.97	13.13	0.59	0.59	9.14	9.25
Laboratory 3	13.97	13.62	0.60	0.60	9.81	9.58
Laboratory 4	12.23	12.07	0.65	0.64	8.69	8.57
Laboratory 5	12.55	12.47	0.56	0.55	8.83	8.77
Laboratory 6	12.68	12.67	0.59	0.61	8.95	8.95
Laboratory 7	13.10	13.44	0.56	0.55	9.20	9.42
Laboratory 8	12.81	13.29	0.56	0.64	9.01	9.39
Laboratory 9	12.59	11.22	0.58	0.52	8.88	7.91
Laboratory 10	14.32	12.47	0.54	0.54	10.01	8.77
Laboratory 11	12.52	12.48	0.59	0.60	8.84	8.82
Laboratory 12	12.42	12.49	0.61	0.62	8.78	8.84
Laboratory 13	14.03	13.98	0.65	0.63	9.89	9.84
Laboratory 14	6.85[a]	6.84[a]	0.99[a]	1.01[a]	5.33[a]	5.33[a]
Laboratory 15	10.18	10.95	0.44	0.47	7.16	7.69

[a] Cochran and/or Grubbs outliers.

Table 14. Collaborative data for infant formula powder partially hydrolyzed soy-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.75	1.76	0.49[a]	0.40[a]	1.53	1.48
Laboratory 2	1.97	1.97	0.49	0.12	1.69	1.41
Laboratory 3	1.93	1.91	0.49[a]	0.07[a]	1.65	1.34
Laboratory 4	1.89	1.87	0.55	0.55	1.68	1.66
Laboratory 5	1.85	1.86	0.48	0.49	1.60	1.62
Laboratory 6	1.93	1.91	0.50	0.47	1.66	1.63
Laboratory 7	2.14	1.90	0.45	0.45	1.77	1.61
Laboratory 8	1.88	1.84	0.51	0.50	1.63	1.60
Laboratory 9	1.30	1.39	0.39	0.39	1.16	1.22
Laboratory 10	1.77	1.76	0.48	0.48	1.54	1.54
Laboratory 11	1.84	1.85	0.49	0.49	1.60	1.60
Laboratory 12	1.98	2.04	0.51	0.51	1.70	1.75
Laboratory 13	1.95	1.72	0.53	0.54	1.70	1.55
Laboratory 14	1.55	1.49	0.82[a]	0.79[a]	1.64	1.58
Laboratory 15	1.48	1.53	0.36	0.37	1.26	1.30

[a] Cochran and/or Grubbs outliers.

Table 15. Collaborative data for SRM 1849a

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	159.60	164.40	34.20	31.50	132.33	133.56
Laboratory 2	181.50	182.30	36.30	37.30	148.58	149.85
Laboratory 3	177.00	173.58	38.44	36.09	147.14	143.11
Laboratory 4	177.10	176.60	41.00	41.80	149.10	149.35
Laboratory 5	170.52	172.62	34.31	38.23	139.74	144.05
Laboratory 6	170.42	177.70	31.57	25.79	137.65	138.27
Laboratory 7	166.42	170.06	36.14	34.52	138.34	139.59
Laboratory 8	177.50	177.66	39.25	41.48	148.07	149.83
Laboratory 9	158.63[a]	136.08[a]	36.43	30.64	133.33[a]	113.93[a]
Laboratory 10	169.36	163.34	34.99	36.42	139.46	136.48
Laboratory 11	171.10	170.44	39.25	41.29	143.78	144.84
Laboratory 12	186.39	173.61	35.97	34.82	151.61	142.19
Laboratory 13	211.37	211.62	46.09	43.25	175.85[a]	173.92[a]
Laboratory 14	145.40	142.57	65.24[a]	58.61[a]	145.72	138.92
Laboratory 15	161.44	159.37	29.85	31.82	130.36[a]	130.42[a]

[a] Cochran and/or Grubbs outliers.

Table 16. Collaborative data for adult nutritional powder low fat

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.70	1.82	0.23[a]	0.13[a]	1.32	1.32
Laboratory 2	1.90	1.94	0.09	0.12	1.34	1.39
Laboratory 3	1.96	1.98	0.11	0.10	1.40	1.41
Laboratory 4	1.79	1.88	0.17	0.19	1.32	1.40
Laboratory 5	1.89	1.81	0.25	0.24	1.46	1.39
Laboratory 6	1.93	1.93	0.05	0.06	1.33	1.33
Laboratory 7	1.71	1.72	0.13	0.13	1.24	1.25
Laboratory 8	1.91	1.97	0.15	0.18	1.40	1.45
Laboratory 9	1.71[a]	0.95[a]	0.11	0.08	1.23[a]	0.70[a]
Laboratory 10	1.84	1.90	0.14	0.14	1.34	1.37
Laboratory 11	1.98	1.96	0.13	0.15	1.42	1.43
Laboratory 12	1.68	1.67	0.10	0.09	1.20	1.19
Laboratory 13	1.95	1.99	0.26	0.26	1.50	1.53
Laboratory 14	1.44	1.45	0.16	0.17	1.08	1.09
Laboratory 15	1.52[a]	1.31[a]	0.07	0.08	1.07	0.94

[a] Cochran and/or Grubbs outliers.

Table 17. Collaborative data for infant elemental powder

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.77	1.75	0.41	0.29	1.49	1.39
Laboratory 2	1.83	1.85	0.37	0.35	1.51	1.50
Laboratory 3	1.88	1.93	0.52	0.40	1.64	1.59
Laboratory 4	1.80	1.84	0.41	0.36	1.51	1.50
Laboratory 5	1.78	1.87	0.40	0.47	1.49	1.60
Laboratory 6	1.78	1.94	0.29	0.38	1.41	1.58
Laboratory 7	1.76	1.91	0.42	0.52	1.49	1.67
Laboratory 8	1.85	1.87	0.40	0.43	1.54	1.57
Laboratory 9	1.76	1.76	0.52	0.42	1.57	1.49
Laboratory 10	1.76	1.83	0.31	0.52	1.41	1.62
Laboratory 11	1.75	1.64	0.31	0.32	1.40	1.34
Laboratory 12	2.00	1.91	0.42	0.48	1.66	1.64
Laboratory 13	1.78	1.85	0.41	0.38	1.49	1.52
Laboratory 14	1.49	1.53	0.84[a]	0.58[a]	1.62	1.46
Laboratory 15	1.57	1.69	0.52	0.47	1.44	1.48

[a] Cochran and/or Grubbs outliers.

Table 18. Collaborative data for infant formula powder milk-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.36	1.36	0.47	0.46	1.26	1.25
Laboratory 2	1.50	1.52	0.47	0.47	1.35	1.36
Laboratory 3	1.51	1.48	0.49	0.49	1.38	1.35
Laboratory 4	1.45	1.45	0.53	0.52	1.36	1.36
Laboratory 5	1.47	1.48	0.52	0.51	1.37	1.37
Laboratory 6	1.43	1.43	0.43	0.45	1.28	1.29
Laboratory 7	1.45	1.42	0.50	0.48	1.34	1.31
Laboratory 8	1.45	1.44	0.50	0.49	1.34	1.33
Laboratory 9	1.07[a]	1.37[a]	0.39[a]	0.50[a]	1.00[a]	1.29[a]
Laboratory 10	1.39	1.37	0.49	0.50	1.29	1.29
Laboratory 11	1.45	1.49	0.49	0.51	1.33	1.38
Laboratory 12	1.55	1.57	0.51	0.51	1.41	1.43
Laboratory 13	1.41	1.48	0.52	0.54	1.33	1.39
Laboratory 14	1.17[a]	1.15[a]	0.82[a]	0.81[a]	1.39	1.36
Laboratory 15	1.30	1.33	0.45	0.45	1.21	1.22

[a] Cochran and/or Grubbs outliers.

Table 19. Collaborative data for infant formula RTF milk-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.49	1.48	0.39	0.36	1.29	1.26
Laboratory 2	1.64	1.62	0.45	0.44	1.43	1.41
Laboratory 3	1.66	1.64	0.47	0.47	1.46	1.44
Laboratory 4	1.55	1.56	0.50	0.50	1.41	1.42
Laboratory 5	1.53	1.53	0.49	0.50	1.39	1.39
Laboratory 6	1.59	1.58	0.42	0.41	1.37	1.37
Laboratory 7	1.57	1.55	0.49	0.49	1.42	1.40
Laboratory 8	1.56	1.55	0.47	0.47	1.39	1.39
Laboratory 9	1.29[a]	1.47[a]	0.43	0.46	1.18[a]	1.32[a]
Laboratory 10	1.49	1.50	0.47	0.47	1.35	1.35
Laboratory 11	1.57	1.57	0.45	0.45	1.39	1.38
Laboratory 12	1.60	1.59	0.43	0.43	1.39	1.38
Laboratory 13	1.65	1.65	0.47	0.43	1.45	1.42
Laboratory 14	1.26[a]	1.28[a]	0.75[a]	0.76[a]	1.40	1.42
Laboratory 15	1.43	1.44	0.43	0.42	1.28	1.28

[a] Cochran and/or Grubbs outliers.

Table 20. Collaborative data for infant formula powder soy-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.27	1.26	0.41	0.40	1.15	1.14
Laboratory 2	1.38	1.40	0.43	0.43	1.25	1.26
Laboratory 3	1.36	1.37	0.45	0.46	1.24	1.26
Laboratory 4	1.33	1.34	0.52	0.40	1.27	1.19
Laboratory 5	1.30	1.33	0.49	0.49	1.23	1.26
Laboratory 6	1.36	1.31	0.46	0.44	1.25	1.20
Laboratory 7	1.33	1.33	0.50	0.49	1.26	1.25
Laboratory 8	1.30	1.31	0.45	0.41	1.21	1.18
Laboratory 9	0.88[a]	1.27[a]	0.35	0.48	0.85[a]	1.21[a]
Laboratory 10	1.28	1.28	0.50	0.49	1.22	1.22
Laboratory 11	1.33	1.32	0.47	0.48	1.24	1.24
Laboratory 12	1.37	1.44	0.46	0.50	1.26	1.33
Laboratory 13	1.30	1.33	0.49	0.51	1.24	1.27
Laboratory 14	1.07	1.09	0.81[a]	0.80[a]	1.32	1.32
Laboratory 15	1.22	1.17	0.44	0.43	1.15	1.11

[a] Cochran and/or Grubbs outliers.

Recommendations

The completed AOAC INTERNATIONAL *Interlaboratory Study Workbook Revision 2.1* along with a statistical report (5) and a draft copy of the study report summarizing the outcomes of this collaborative study were submitted with the recommendation that AOAC First Action Method **2012.10** be accepted as a SPIFAN-endorsed AOAC Final Action Method. The ERP evaluated the collaborative study data in September 2014 and endorsed the recommendation, which was subsequently approved by the Official Methods Board in November 2014.

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(3) *Official Methods of Analysis* (2012) *Appendix F: Guidelines for Standard Method Performance Requirements*, 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD

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INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Labeled Fatty Acids Content in Milk Products, Infant Formula, and Adult/Pediatric Nutritional Formula by Capillary Gas Chromatography: Collaborative Study, Final Action 2012.13

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A collaborative study was conducted on AOAC First Action Method 2012.13 "Determination of Labeled Fatty Acids Content in Milk Products and Infant Formula by Capillary Gas Chromatography," which is based on an initial International Organization for Standardization (ISO)–International Dairy Federation (IDF) New Work Item that has been moved forward to ISO 16958:2015 | IDF 231:2015 in November 2015. It was decided to merge the two activities after the agreement signed between ISO and AOAC in June 2012 to develop common standards and to avoid duplicate work. The collaborative study was performed after having provided highly satisfactory single-laboratory validation results [Golay, P.A., & Dong, Y. (2015) *J. AOAC Int.* 98, 1679–1696] that exceeded the performance criteria defined in AOAC *Standard Method Performance Requirement* (SMPR®) 2012.011 (September 29, 2012) on 12 products selected by the AOAC Stakeholder Panel on Infant Formula (SPIFAN). After a qualification period of 1 month, 18 laboratories participated in the fatty acids analysis of 12 different samples in duplicate. Six samples were selected to meet AOAC SPIFAN requirements (i.e., infant formula and adult nutritionals in powder and liquid formats), and the other Six samples were selected to meet ISO-IDF requirements (i.e., dairy products such as milk powder, liquid milk, cream, butter, infant formula with milk, and cheese). The fatty acids were analyzed directly in all samples without preliminary fat extraction, except in one sample (cheese). Powdered samples were analyzed after dissolution (i.e., reconstitution) in water, whereas liquid samples (or extracted fat) were analyzed directly. After addition of the internal standards solution [C11:0 fatty acid methyl ester (FAME) and C13:0 triacylglycerols (TAG)] to the samples, fatty acids attached to lipids were transformed into FAMEs by direct transesterification using methanolic sodium methoxide. FAMEs were separated using highly polar capillary GLC and were identified by comparison with the retention times of pure analytical standards. Quantification of fatty acids was done relative to C11:0 FAME as internal standard and to instrument response factors (determined separately using calibration standards mixture). The performance of the method (i.e., transesterification) was monitored in all samples using the second internal standard, C13:0 TAG. RSD_R values were summarized separately for labeled fatty acids in SPIFAN materials and ISO-IDF materials due to different expression of results. This method was applied to representative dairy, infant formula, and adult/pediatric nutritional products and demonstrated global acceptable reproducibility precision for all fatty acids analyzed (i.e., 46 individuals and/or groups) for these categories of products.

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The method was approved by the AOAC Official Methods Board as Final Action. *See* "Standards News," (2014) *Inside Laboratory Management*, November/December 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.

Appendixes are available on the *J. AOAC Int*. website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac

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It is well known that fatty acids play an important role in human nutrition at all periods of life. Some fatty acids are considered more desirable than others (i.e., essential fatty acids), and some, like the saturated fatty acids (SFAs) and the industrial *trans* fatty acids (TFAs), need to be decreased and limited in foods due to their potential contributions to cardiovascular diseases. Fatty acids are naturally present in oils and fats used as raw materials but in different concentrations. As a consequence, they are also present in manufactured food products for which strict nutritional recommendations and/or regulation are sometimes given according to the target population.

To support the labeling of fatty acids, the food industry (as well as governmental laboratories) needs reliable and horizontal methods for analyzing the whole fatty acids spectrum, including TFAs. To address this need, a method involving direct preparation of fatty acid methyl esters (FAMEs) using a high-resolution

chromatographic capillary column of 100 m long has been developed for its use in various laboratories using different GC equipment and different types of injectors. The response factors of the equipment have been taken into account in the calculations to provide quantitative fatty acids results. The method has already been implemented in several laboratories, and their performance has been regularly evaluated and monitored via proficiency tests. The method was also published in a scientific paper (1) before being proposed for the standardization process, first with the International Organization for Standardization (ISO)–International Dairy Federation (IDF) as a New Work Item proposal moved forward to an International Standard (2). In view of the absence of an internationally recognized analytical method for fatty acids in the selected SPIFAN matrixes, the method proposed to ISO-IDF was identified as a good candidate to meet AOAC *Standard Method Performance Requirement* (SMPR®) 2012.011 (3, 4) defined by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN).

In the frame of the agreement between ISO and AOAC to develop common standards (signed June 18, 2012), it was decided to merge the two activities to avoid duplicate work.

To verify the method performance for SPIFAN-selected matrixes, a single-laboratory validation (SLV) study was performed on all samples (SPIFAN test kit of 12 samples).

After evaluation of the SLV data, an AOAC Expert Review Panel (ERP) determined that the method met SMPR 2012.011 as approved by SPIFAN. The ERP granted the method Official First Action status on October 2, 2012 (5) and SLV results were then published separately (6). The method was recommended to advance to multilaboratory collaborative study for the evaluation of reproducibility (7).

Collaborative Study

Although more than 30 laboratories initially indicated their potential interest for involvement in this study, some were not considered principally due to their location, difficulties in shipping dairy samples, and time or resource constrains. The final enrollment of participating laboratories, which included food manufacturers, food regulatory agencies, food research institutes, and private laboratories, was decided after satisfactory results were shown on the training sample (a milk powder used also in the collaborative study), and satisfactory chromatographic resolution between C18:1 *cis* and *trans* isomers, which is essential for the accurate determination of TFAs in dairy products. The six samples selected by SPIFAN were shipped to participants from Covance Laboratories (Madison, WI), and the six other samples selected by ISO-IDF were shipped to participants from Nestlé (Lausanne, Switzerland) are listed in Table 1. Each participant recorded data on a single template that contained sections for reporting all raw data and fatty acid calculations and for including chromatograms and comments.

Method

The protocol was based on AOAC First Action Method **2012.13** for analyzing infant formula and adult/pediatric nutritional products and on the ISO-IDF method for analyzing dairy matrixes (i.e., cheese sample).

AOAC Official Method 2012.13
Determination of Labeled Fatty Acids Content in Milk Products, Infant Formula (and Adult/Pediatric Nutritional Formula)
Capillary Gas Chromatography
First Action 2012
Final Action 2014

ISO/IDF-AOAC Method

A. Scope

The method is applicable to the determination of all fatty acids, including individual labeled fatty acids [i.e., linoleic acid (LA), α-linoleic acid (ALA), arachidonic acid (ARA), ecosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)] and/or group of fatty acids [i.e., trans fatty acids (TFAs), saturated fatty acids (SFAs), nonounsaturated fatty acids (MUFAs), polyunsaturated fatty acid (PUFAs), omega-3,

Table 1. Collaborative study samples and codes

Sample No.[a]	Product	Fat, %	Sample A MLT code[b]	Sample B MLT code
1	Full cream (milk powder)	26.27	GHXZ007	SJLO002
2	Full cream (liquid milk)	3.55	JSYB023	GPOQ091
3	Full cream	35.27	KLMQ050	SYKA045
4	Butter	82.93	DDHU078	UYBE089
5	Cheese (soft)	13.29	MJFR034	WHTF002
6	Infant formula (powder)	25.67	SZEC013	VCIN029
7	Adult nutritional (milk-protein powder)	17.44	LARU224	GLVC238
8	Infant formula (partially hydrolyzed soy powder)	26.01	LUJP087	ADVZ021
9	Infant formula (milk-based powder)	28.38	YKLP059	ZNPI092
10	Infant formula RTF (milk-based liquid)	3.57	MOPG098	SJLQ035
11	Adult nutritional RTF (high-protein liquid)	3.58	LHTK069	LKAU043
12	Adult nutritional RTF (high-fat liquid)	8.61	VFJL091	YATV077

[a] Sample Nos. 1 to 6 were selected by ISO-IDF and shipped from Nestlé (Lausanne, Switzerland); Sample Nos. 7 to 12 were selected by SPIFAN and shipped from Covance (Madison, WI). Analysis was performed on duplicate samples (A and B).

[b] MLT = Multilaboratory testing.

omega-6, and omega-9] in milk products, infant formula, and adult/pediatric nutritional formula containing milk fat and/or vegetable oils, supplemented or not supplemented with oils rich in long-chain PUFAs.

The determination is performed by direct transesterification of food samples without prior fat extraction and, consequently, it is applicable to liquid samples or reconstituted powder.

Products containing <1.5% fat can be analyzed after preliminary fat extraction using a suitable fat-extraction reference method (i.e., ISO/IDF-AOAC).

In the case of products supplemented or enriched with PUFAs having fish-oil or algae origins, the extraction solvents must be evaporated at a maximum of 40°C.

B. Principle

Addition of the internal standard solution to the sample, preparation of FAMEs by direct transesterification with methanolic sodium methoxide for liquid samples and fat extracted from food; dissolution (i.e., reconstitution) in water for powder sample and direct transesterification with methanolic sodium methoxide. Separation of FAMEs using capillary GLC. Identification of peak by comparison with the retention time of pure standards and quantification as fatty acids by reference to an internal standard (C11:0 FAME) and instrument response factors. Verification of the transesterification performance using a second internal standard [C13:0 triacylglycerols (TAG)].

C. Apparatus and Materials

Common laboratory equipment and, in particular, the following:

(a) *Analytical balance*.—Capable of weighing to the nearest 1 mg, with a readability of 0.1 mg.

(b) *One-mark volumetric flasks*.—50, 100, 250, 300, and 500 mL.

(c) *One-mark volumetric pipets*.—2, 5, 10, 25, and 50 mL; class AS (ISO).

(d) *Two-mark pipet, volumetric*.—2 and 5 mL; class AS (ISO).

(e) *Micropipet*.—200 µL.

(f) *Dispensers*.—2, 5, and 10 mL.

(g) *Test tube*.—26 mm (diameter) × 100 mm (length), fitted with PTFE-lined screw cap.

(h) *Test tube mixer*.—Vortex Genie Scientific Industries, Inc., Bohemia, NY, or equivalent.

(i) *Laboratory centrifuge*.—Equipped with adapters for test tubes with external diameter of 26 mm.

(j) *Gas–liquid chromatograph*.—Equipped with flame ionization detector and capillary split-injection system or on-column. Autosampler and integration system preferably computerized.

Note: Use of the cleanest possible glassware and caps is required to avoid impurities in the FAME chromatogram.

(*1*) *Carrier gas*.—Hydrogen or helium. Purity ≥99.9997%.

Note: The use of hydrogen or helium affects principally the chromatography duration but does not have any significant impact on the chromatographic resolution.

(*2*) *Other gases*.—Free from organic impurities (C_nH_m <1 ppm), nitrogen and hydrogen, purity at least ≥99.995%, and compressed pure air.

(*3*) *Capillary column*.—Cyanopropyl-polysiloxane phase (or equivalent polarity) capillary columns with 100 m length × 0.25 mm id, 0.2 µm film thickness.

Note: Traces of oxygen and humidity will damage the polar phase of the column. When pure gas is not available, use a gas purifying filter device.

D. Chemicals and Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

(a) *Water*.—HPLC grade or equivalent quality.

(b) *Sodium methoxide solution (CH_3ONa)*.—Dissolved in methanol 30% (w/v; ca 5.4 M).

(c) *Transesterification solution*.—Sodium methoxide solution 5% in methanol. Into a 300 mL volumetric flask, pipet 50 mL sodium methoxide solution, **D**(**b**), and complete gently with 250 mL methanol using a magnetic stirrer. Remove the magnetic stirrer, then cool to room temperature, and dilute to the mark with methanol. Stored in the dark at 4°C, this solution is stable for 1 week. Allow the solution to come to room temperature before use. Perform the transesterification reaction at ambient temperature (20–25°C).

(d) *Disodium hydrogen citrate sesquihydrate [$HOC(COOH)(CH_2COONa)_2{\cdot}1.5H_2O$]*.

(e) *Sodium chloride (NaCl)*.—Puriss.

(f) *Neutralization solution*.—Disodium hydrogen citrate sesquihydrate 10%, sodium chloride 15% in water. Weigh 50.0 g disodium hydrogen citrate sesquihydrate, **D**(**d**), and 75.0 g sodium chloride, **D**(**e**), in a 500 mL volumetric flask, **C**(**b**). Dissolve in 450 mL water using a magnetic stirrer. Remove the magnetic stirrer, and dilute to volume with water. Stored in the dark at 4°C, this solution is stable for 1 month. Salt crystals may appear in the solution during storage but disappear after shaking. Allow the solution to come to room temperature before use.

(g) *tert-Butyl methyl ether (MTBE)*.

(h) *Methyl undecanoate (C11:0 FAME)*.—Purity ≥99% mass fraction.

(i) *Tritridecanoin (C13:0 TAG)*.—Purity ≥99% mass fraction.

(j) *C11:0 FAME/C13:0 TAG standard solution*.—Into a 250 mL volumetric flask, weigh to the nearest 0.1 mg about 500 mg tritridecanoin, **D**(**i**), and 500 mg methyl undecanoate, **D**(**h**). Dissolve and dilute to the mark with MTBE. Stored in the dark at 4°C, this solution is stable for 1 wk. Allow the solution to come to room temperature before use.

(k) *Octadecenoic acid methyl ester*.—*Cis/trans* isomer mixture of C18:1 with *trans*-4 to *trans*-16 (all isomers) and principal *cis* isomers. Concentration 2.5 mg/mL in methylene chloride.

Note: This standard is commercially available from the Supelco brand of Sigma-Aldrich St. Louis, MO (Cat. No. 40495-U).

(l) *Linoleic acid methyl ester*.—*Cis/trans* isomer mixture of C18:2 with *trans*-9, *trans*-12-octadecadienoic acid (50%); *cis*-9, *trans*-12-octadecadienoic acid (20%); *trans*-9, *cis*-12-octadecadienoic acid (20%); and *cis*-9, *cis*-12-octadecadienoic acid (10%). Concentration 10 mg/mL in methylene chloride.

Note: This standard is commercially available from the Supelco brand of Sigma-Aldrich (Cat. No. 47791).

(m) *Linolenic acid methyl ester*.—*Cis/trans* isomer mixture of C18:3 with *cis*-9, *cis*-12, *cis*-15-octadecatrienoic acid methyl ester [ca 3% (w/w)]; *cis*-9, *cis*-12, *trans*-15-octadecatrienoic acid methyl ester [ca 7% (w/w)]; *cis*-9, *trans*-12, *cis*-15-octadecatrienoic acid methyl ester [ca 7% (w/w)]; *cis*-9, *trans*-12, *trans*-15-octadecatrienoic acid methyl ester [ca 15% (w/w)]; *trans*-9, *cis*-12, *cis*-15-octadecatrienoic acid methyl ester [ca 7% (w/w)]; *trans*-9, *cis*-12, *trans*-15-octadecatrienoic acid methyl ester [ca 15% (w/w)]; *trans*-9, *trans*-12, *cis*-15-octadecatrienoic acid methyl ester [ca 15% (w/w)]; and *trans*-9, *trans*-12, *trans*-15-octadecatrienoic acid methyl ester [ca 30% (w/w)]. Concentration 10 mg/mL in methylene chloride.

Note: This standard is commercially available from the Supelco brand of Sigma-Aldrich (Cat. No. 47792). This standard contains all *trans* isomers (eight in total) but their abundance and ratio are different from those observed in deodorized oils and fats.

(n) *Methyl octadecadienoate conjugated acids*.—Mixture of C18:2 *cis*-9, *trans*-11 and *cis*-10, *trans*-12; purity ≥99%.

(o) *Qualitative cis/trans FAME isomers standard mixture solution*.—For the Retention Time (RT) identification of *cis/trans* isomers, prepare a qualitative standard solution with the standard listed, **D(k)**–**D(n)**. All standards commercially available could be used. Into a 50 mL volumetric flask, add each standard isomer in equal proportion. Dissolve and dilute to the mark with hexane. Dilute according to the type of GC injector used.

(p) *Standard FAME mixture solution*.—Quantitative FAME standard mixture (Nu-Check-Prep, Cat. No. GLC-Nestle36) containing butyric acid methyl ester (C4:0); caproic acid methyl ester (C6:0); caprylic acid methyl ester (C8:0); capric acid methyl ester (C10:0); undecanoic acid methyl ester (C11:0); lauric acid methyl ester (C12:0); tridecanoic acid methyl ester (C13:0); myristic acid methyl ester (C14:0); myristoleic acid methyl ester (C14:1 *n*-5 *cis*); pentadecanoic acid methyl ester (C15:0); *cis*-10-pentadecenoic acid methyl ester (C15:1 *n*-5 *cis*); palmitic acid methyl ester (C16:0); palmitoleic acid methyl ester (C16:1 *n*-7 *cis*); heptadecanoic acid methyl ester (C17:0); *cis*-10-heptadecenoic acid methyl ester (C17:1 *n*-7 *cis*); stearic acid methyl ester (C18:0); elaidic acid methyl ester (C18:1 *n*-9 *trans*); oleic acid methyl ester (C18:1 *n*-9 *cis*); linolelaidic acid methyl ester (C18:2 *n*-6 *trans*); linoleic acid methyl ester (C18:2 *n*-6 *cis*); arachidic acid methyl ester (C20:0); gamma-linoleic acid methyl ester (C18:3 *n*-6 gamma); *cis*-11-eicosenoic acid methyl ester (C20:1 *n*-9 *cis*); linolenic acid methyl ester (C18:3 *n*-3 *cis*); *cis*-11,14-eicosadienoic acid methyl ester (C20:2 *n*-6 *cis*); behenic acid methyl ester (C22:0); *cis*-8,11,14-eicosatrienoic acid methyl ester (C20:3 *n*-6 *cis*); erucic acid methyl ester (C22:1 *n*-9 *cis*); *cis*-11,14,17-eicosatrienoic acid methyl ester (C20:3 *n*-3 *cis*); arachidonic acid methyl ester (C20:4 *n*-6 *cis*); *cis*-13,16-docosadienoic acid methyl ester (C22:2 *n*-6 *cis*); lignoceric acid methyl ester (C24:0); *cis*-5,8,11,14,17-eicosapentanoic acid methyl ester (C20:5 *n*-3 *cis*); nervonic acid methyl ester (C24:1 *n*-9 *cis*); and *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester (C22:6 *n*-3 *cis*).

Note: It is also possible to prepare the FAME standard mixture from individual and pure FAME standards, but the purchasing of individual FAME standards is more expensive and the preparation is time consuming and requires high precision.

The weight percentage of each FAME component is indicated in the accompanying certificate. Each ampoule contains ca 100 mg of the FAME calibration standard mix. All individual FAMEs are distributed in equal proportions in the standard, except for palmitic acid methyl ester (C16:0) in double amount.

(q) *Preparation of calibration standard FAME mixture solution*.—Before use, allow the ampoule, **D(p)**, to come to room temperature (maximum of 25°C) in the dark without heating. Cut the ampoule with a glass knife and using a Pasteur pipet, rapidly transfer the content of the ampoule into a 50 mL pretarred volumetric flask, weigh, and dilute to the mark with *n*-hexane. Dilute accordingly to the type of injector used.

Note: These solutions keep for about 6 months when stored in the dark at –20°C.

E. Sample Preparation

(a) *Milk product, infant formula, and adult/pediatric nutritional*.— Mix well to ensure that sample is homogeneous.

(b) *Test portion*.—Into a 25 mL centrifuge tube with a screw cap, weigh to the nearest 0.1 mg an equivalent quantity of sample to obtain ca 50 mg fat in the tube (*Example*: for a sample containing 26 g fat/100 g product, the corresponding sample weight is approximately 190 mg).

Note: For fatty acid analysis on fat extracted from foods, the same amount of fat is required (about 50 mg). For milk powder or infant formula powder, add 2.0 mL water using a micropipet. Close the tube, and then dissolve gently using a vortex mixer. Wait for 15 min at room temperature.

Note: For liquid milk samples and fat extracted from foods, no pretreatment (water addition) is required.

Pipet 5 mL internal standard solution, **D(j)**. Add with a pipet 5 mL 5% (w/v) methanolic sodium methoxide solution, **D(c)**. The transesterification time starts with the addition of the first drop of reagent, **D(c)**. Close the tube hermetically and shake well for 10 s using a vortex mixer. After 180 s, open the tube and add 2 mL hexane. After 210 s, add 10 mL disodium hydrogen citrate and sodium chloride aqueous solution, **D(f)**. The transesterification time stops after the addition of the last drop of neutralization solution, **D(f)**. Shake gently using a vortex mixer for 30 s. The transesterification time should not exceed 240 s. Centrifuge the tube at 1750 rpm (or equivalent rpm to $g = 375 \pm 25$) for 5 min.

Into a 10 mL volumetric flask, pipet 200 µL supernatant and dilute to the mark with *n*-hexane.

Note: The dilution factor is calculated for on-column injection only. When using split injection, adapt the dilution to obtain the desired peak responses according to split ratio used (ensure sufficient and accurate detection level for small peaks especially). Stored in the dark at 4°C, the sample solution after dilution is stable for 2 days.

F. Chromatography Analysis

(a) *Gas GC conditions*.—The oven temperature and the carrier gas flow depend on the column selected and the carrier gas adopted (i.e., hydrogen or helium). In any case, the selected conditions must allow the separation between *cis* and *trans* zone for C18:1, C18:2, C18:3, and conjugated linoleic acid (CLA) (Figures **2012.13A** and **2012.13B**). For the accurate quantification of C18:1 TFA (level ≥0.5 g/100 g fat), a sufficient resolution between C18:1 *trans*-13/14 and C18:1 *cis*-9 is required. The resolution is determined with the injection of the

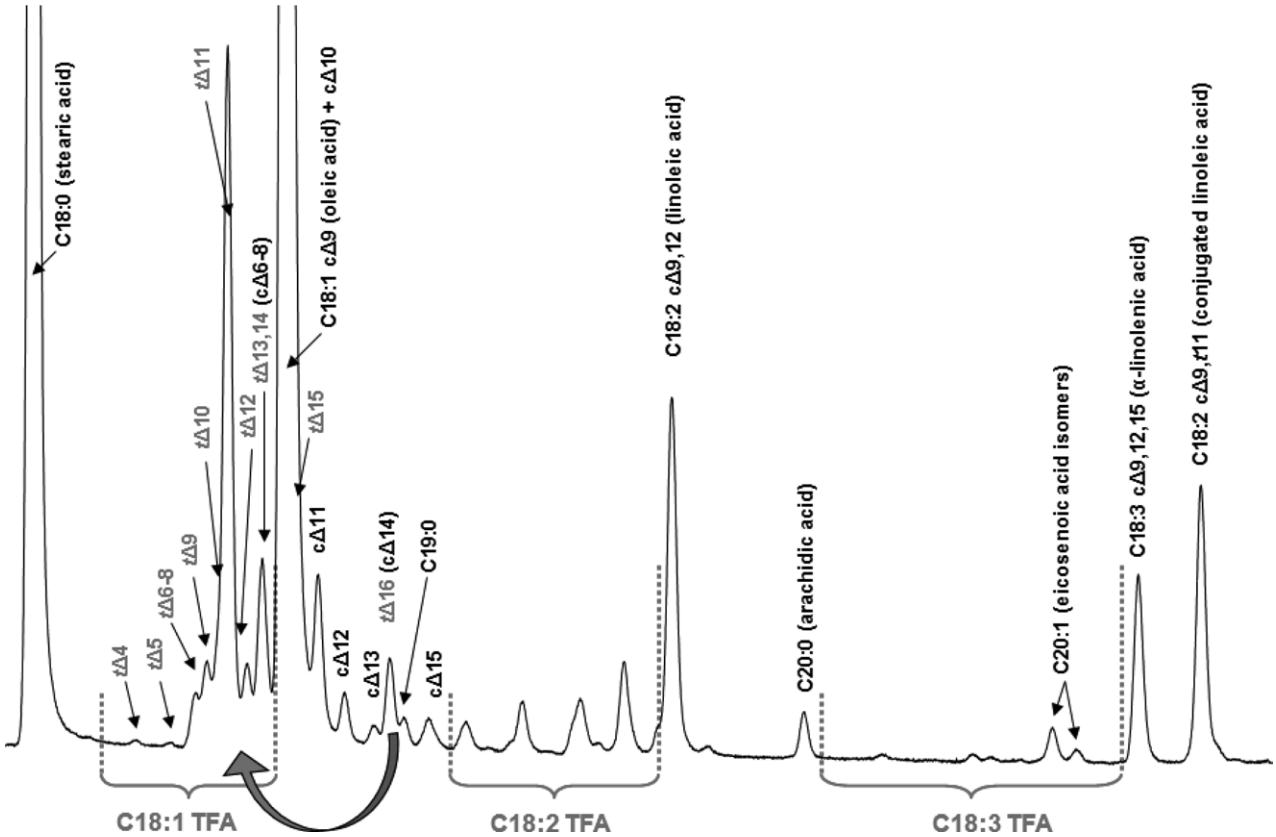

Figure 2012.13A. Example of a GC chromatogram (enlarged view of C18:1 TFA, C18:2 TFA, and CLA) using split injection.

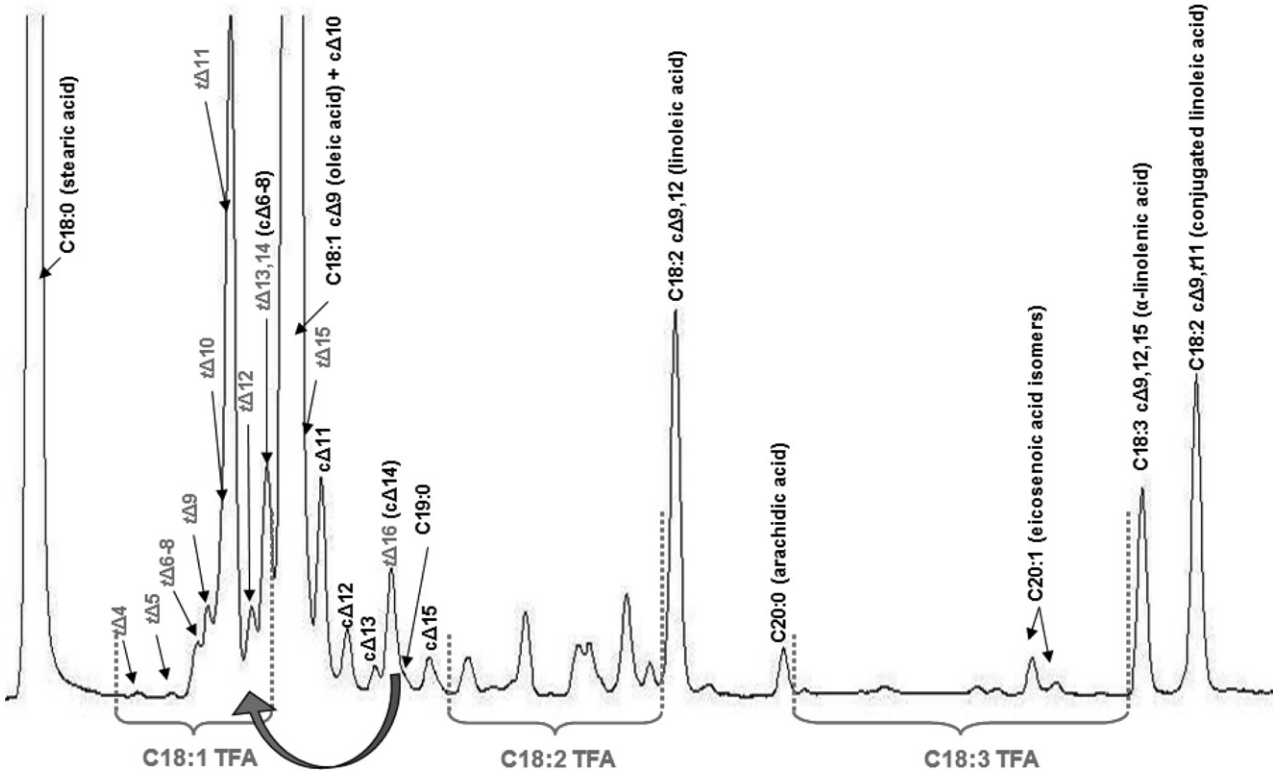

Figure 2012.13B. Example of a GC chromatogram (enlarged view of C18:1 TFA, C18:2 TFA, C18:3 TFA, and CLA) using on-column injection.

qualitative *cis/trans* C18:1 FAME isomers standard mixture solution, **D**(**k**). The resolution is sufficient when resolution (R) criteria is equivalent or higher than 1.00 (Figure **2012.13C**).

The following two examples report applicable conditions for a correct separation of *cis* and *trans* with different GC injectors.

(b) *Example 1.*—Split injection mode.

(*1*) *Column.*—100 m length × 0.25 mm id, 0.2 µm film thickness, fused silica capillary column.

(*2*) *Stationary phase.*—Cyanopropyl-polysiloxane or equivalent polarity.

(*3*) *Carrier gas type.*—Helium.

(*4*) *Column head carrier gas pressure.*—225 KPa (175–225 KPa).

(*5*) *Split flow.*—25.5 mL/min.

(*6*) *Split ratio.*—10:1.

(*7*) *Injector temperature.*—250°C.

(*8*) *Detector temperature.*—275°C.

(*9*) *Oven temperature program.*—Initial temperature of 60°C, maintained for 5 min, raised at a rate of 15°C/min up to 165°C, maintained at this temperature for 1 min, and then raised at a rate of 2°C/min up to 225°C for 20 min.

(*10*) *Amount of sample injected.*—1.0 µL.

An example of the GC profile obtained with these conditions is reported in Figure **2012.13A**.

(c) *Example 2.*—On-column injection mode.

(*1*) *Column.*—100 m length × 0.25 mm id, 0.2 µm film thickness, fused silica capillary column.

(*2*) *Stationary phase.*—Cyanopropyl-polysiloxane or equivalent polarity.

(*3*) *Carrier gas type.*—Hydrogen.

(*4*) *Column head carrier gas pressure.*—210 KPa (175–225 KPa).

(*5*) *Injector temperature.*—Cold.

(*6*) *Detector temperature.*—275°C.

(*7*) *Oven temperature program.*—Initial temperature of 60°C, maintained for 5 min, raised at a rate of 15°C/min up to 165°C, maintained at this temperature for 1 min, and then raised at a rate of 2°C/min up to 225°C for 17 min.

(*8*) *Amount of sample injected.*—1.0 µL.

An example of the GC profile obtained with these conditions is reported in Figure **2012.13B**.

(d) *Resolution between C18:1 trans and* cis.—Inject into the gas chromatograph 1.0 µL calibrating solution, **D**(**k**). Determine peak width at half height and distance between the left of the

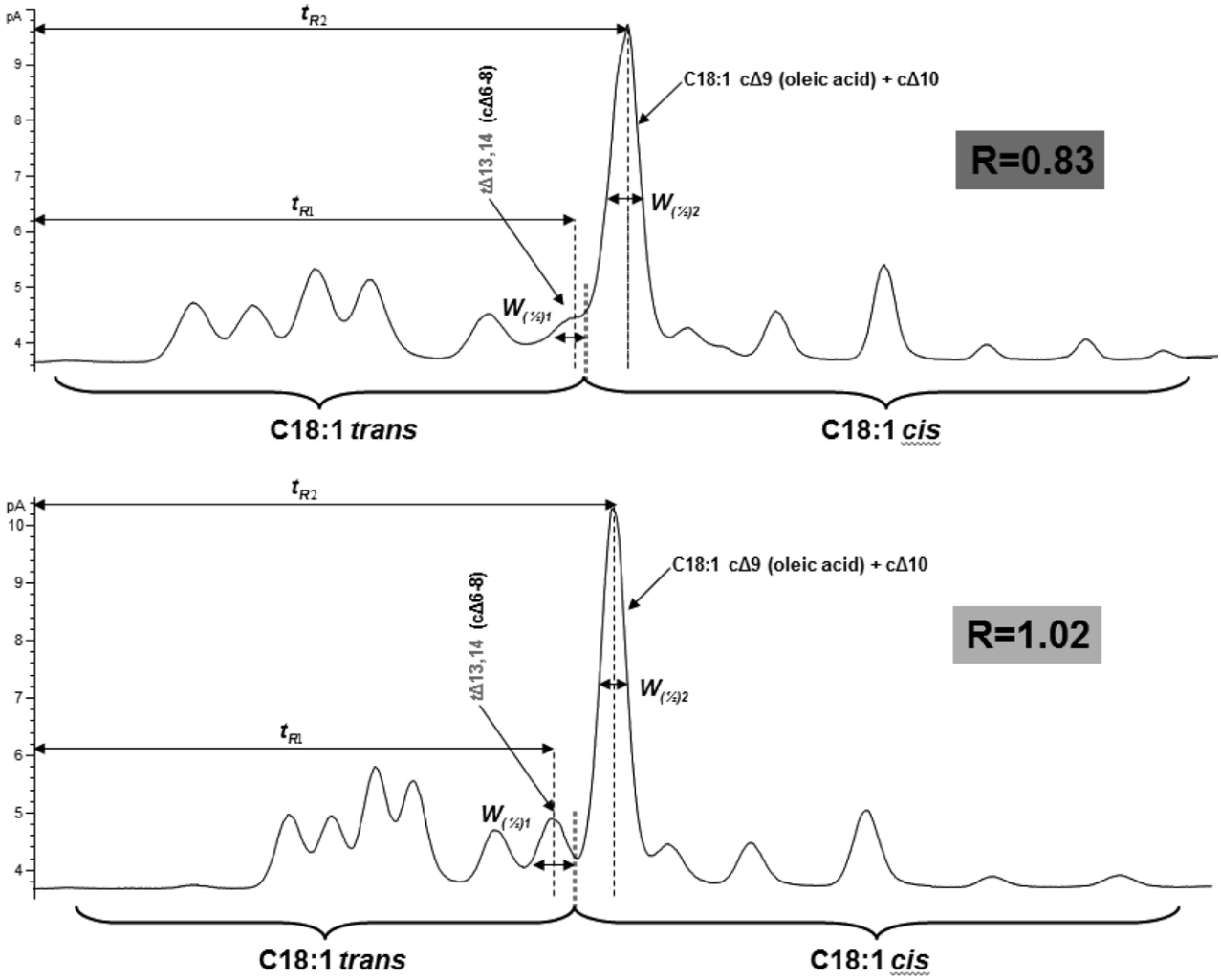

Figure 2012.13C. Example of a GC chromatogram (i.e., with insufficient and sufficient resolution for C18:1 *trans*).

chromatogram and the top of peak for C18:1 *trans*-13/14 and C18:1 *cis*-9 (oleic acid methyl ester). R is calculated as follows:

$$R = 1.18\left(t_{R2} - t_{R1}\right) / W_{\left(\frac{1}{2}\right)1} + W_{\left(\frac{1}{2}\right)2}$$

where t_{R1} = distance in centimeters between the left of the chromatogram and the top of peak 1 (C18:1 *trans*-13/14), t_{R2} = distance in centimeters between the left of the chromatogram and the top of peak 2 (C18:1 *cis*-9), $W_{(1/2)1}$ = peak width in centimeters at half height of peak 1 (C18:1 *trans*-13/14), and $W_{(1/2)2}$ = peak width in centimeters at half height of peak 2 (C18:1 *cis* -9).

The resolution is sufficient when resolution (R) criteria is equivalent or higher than 1.00 (Figure 2012.13C)

Note: In the case of insufficient resolution but with R close to the target value, the fine tuning of chromatography conditions (i.e., slight modification of carrier-gas pressure/flow, oven temperature program) can give an acceptable R value.

(e) *Calibrating solution for the determination of response factor.*—Inject into the gas chromatograph three times 1.0 µL calibrating solution, **D**(**q**).

(f) *Determination of the test portion.*—Inject 1 µL test portion, **E**(**b**), into the gas chromatograph, applying the same conditions as used for the calibrating solution.

(g) *Fatty acid identification.*—Identify the fatty acids in the sample-solution chromatogram by comparing their retention times with those of the corresponding peaks in the calibration standard solution, **D(q)**, and in the qualitative standard mixture containing TFAs and CLA, **D**(**o**).

(*1*) *C18:1 TFA.*—Identify and group all *trans* isomers of C18:1 (include also the peak area of C18:1 *trans*-16 eluted in the C18:1 *cis* region just after the oleic acid methyl ester) according to Figures **2012.13A** and **2012.13B**.

Note: When milk fat is present, two *trans* isomers of C18:1 are eluted in the C18:1 *cis* region (the C18:1 *trans*-15 and C18:1 *trans*-16, respectively), but only one isomer is resolved (C18:1 *trans*-16) with the 100 m long capillary column. The other isomer (C18:1 *trans*-15) is generally overlapped with the oleic acid peak (C18:1 *cis*-9), and its area is quantifiable only by using a preliminary separation (i.e., TLC Ag^+, HPLC Ag^+) followed by a capillary GC analysis. It has been demonstrated that there is no significant difference in total C18:1 *trans* amount when the area of C18:1 *trans*-15 (the not-resolved peak) is not included in the sum in comparison to the result obtained after preliminary separation techniques followed by a capillary GLC analysis (1). A part of this phenomenon is due to the presence of C18:1 *cis* 6-8 isomers within the zone of elution of C18:1 *trans* (1).

(*2*) *C18:2 TFA.*—Identify and group all *trans* isomers of linoleic acid (Figures **2012.13A**, and **2012.13B** and **2012.13D**). For the total TFA of C18:2, include all the *trans* isomers present in milk fat sample as shown in Figures **2012.13A** and **2012.13B**.

(*3*) *C18:3 TFA.*—Identify and group all *trans* isomers of linolenic acid (Figures **2012.13A**, and **2012.13B** and **2012.13D**).

Note: In the presence of milk fat and/or fish oil in the sample, another isomer of C20:1 elutes just before C20:1 *n*-9 (or C20:1 *cis*-11). Depending on the column resolution, the retention time of this fatty acid may also correspond to a *trans* isomer of C18:3 *n*-3 (C18:3 *cis*-9, *trans*-12, *cis*-15 or C18:3 *trans*-9, *cis*-12, *cis*-15). When there is only one peak in the corresponding zone of C18:3 TFA, its correct identification corresponds to a C20:1 isomer. When two, three, or four peaks are encountered in the corresponding zone for C18:3 TFA, each peak area should be included in the total areas of C18:3 TFA (*see* elution order and formation rules discussed later). Interferences may also be observed between C18:3 TFA isomers (C18:3 *cis*-9, *cis*-12, *trans*-15; *cis*-9, *trans*-12, *cis*-15; or *trans*-9, *cis*-12, *cis*-15) and C20:1 *n*-9 (or C20:1 *cis*-11). The C20:1 *n*-9 (or C20:1 *cis*-11) can elute with C18:3 *cis*-9, *trans*-12, *cis*-15 (the minor C18:3 *trans* isomer), but its contribution to total C18:3 TFAs is negligible. However, when C20:1 *n*-9 (or C20:1 *cis*-11) shows interferences from C18:3 *cis*-9, *cis*-12, *trans*-12 or with C18:3 *trans*-9, *cis*-12, *cis*-15, the chromatography conditions should be slightly modified to obtain sufficient separation. Interference is also visible when the wrong ratio between C18:3 *cis*-9, *cis*-12, *trans*-15 and C18:3 *trans*-9, *cis*-12, *cis*-15 is observed (the ratio between these isomers is always close to 5:4).

The kinetics of C18:3 *trans* isomers formation in refined and deodorized oils has been analyzed using a highly polar capillary column and is well described in the literature. Kinetics analysis could be used as a confirmatory tool to verify the presence of C18:3 TFA isomers. Most of the time, a maximum number of four *trans* isomers is encountered.

Case 1. Absence of C18:3 TFA isomers.—No peak (if only one peak is detected, *see* the previous *Note* regarding the presence of another C20:1 isomer in milk; the presence of a single C18:3 *trans* isomer is not possible).

Case 2. Presence of C18:3 TFA isomers (a minimum of two isomers: C18:3 cis-9, cis-12, trans-15 and C18:3 trans-9, cis-12, cis-15).—The peak area of C18:3 *trans*-9, *cis*-12, *cis*-15 is ca 80% of the peak area of C18:3 *cis*-9, *cis*-12, *trans*-15 (ratio 5:4). This ratio is always constant when other C18:3 *trans* isomers are present.

Case 3. Presence of C18:3 TFA isomers (three isomers: C18:3 cis-9, cis-12, trans-15; C18:3 cis-9, trans-12, cis-15; and C18:3 trans-9, cis-12, cis-15).—The same as described for Case 2, but with the presence of C18:3 *cis*-9, *trans*-12, *cis*-15. The peak area of this isomer is always small and sometimes below the LOQ. In the case of coelution with C20:1 *n*-9 (C20:1 *cis*-9) or another C20:1 isomer, its contribution to total C18:3 TFAs is negligible.

Case 4. Presence of C18:3 TFA isomers (four isomers: C18:3 trans-9, cis-12, trans-15; C18:3 cis-9, cis-12, trans-15; C18:3 cis-9, trans-12, cis-15; and C18:3 trans-9, cis-12, cis-15).—The same as described in Cases 2 and 3, but with the presence of C18:3 *trans*-9, *cis*-12, *trans*-15. This isomer is formed by the partial degradation of C18:3 *cis*-9, *cis*-12, *trans*-15 and C18:3 *trans*-9, *cis*-12, *cis*-15 (the first two C18:3 *trans* isomers occurred in deodorized vegetable oils). When its amount is high (i.e., >50% of the peak area of C18:3 *cis*-9, *cis*-12, *trans*-15), the presence of other C18:3 *trans* isomers could be suspected, indicating abnormal oil deodorization conditions (i.e., high temperature and/or time). The presence of other C18:3 *trans* isomers can be confirmed with the qualitative standard mixture, **D**(**m**) or **D**(**o**).

Use the following terms to express TFA results:

C18:1 TFA.—The sum of *trans* positional isomers from C18:1 (i.e., from *trans*-4 to *trans*-16)

C18:2 TFA.—The sum of *trans* isomers from C18:2 in deodorized oils (i.e., C18:2 *trans*-9, *trans*-12; C18:2 *cis*-9, *trans*-12; and C18:2 *trans*-9, *cis*-12) and in milk fat (i.e., C18:2 *cis*-9, *trans*-13; C18:2 *trans*-8, *cis*-12; and C18:2 *trans*-11, *cis*-15).

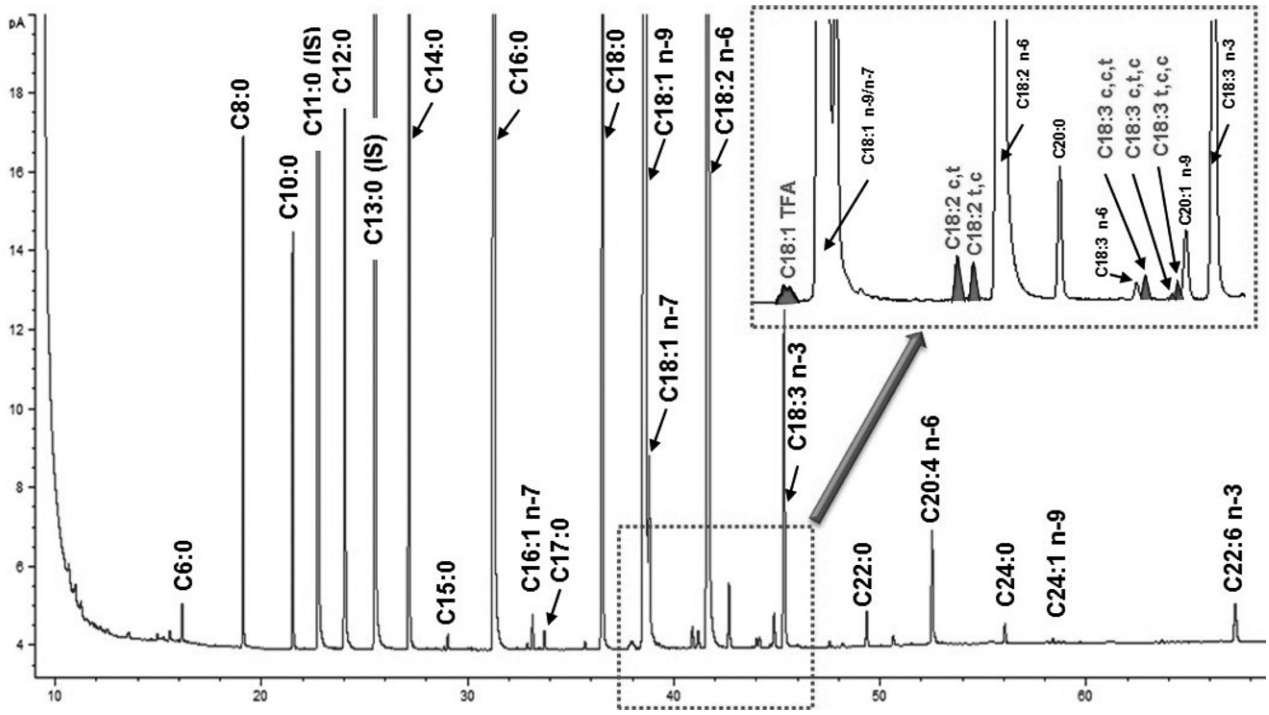

Figure 2012.13D. Example of GC chromatogram of the SPIFAN matrix sample infant formula powder, milk-based. Monounsaturated fatty acids and PUFAs are indicated, counting from the terminal methyl carbon toward the carbonyl carbon (designated as n or ω).

C18:3 TFA.—The sum of *trans* isomers from C18:3 in deodorized vegetable oils (i.e., C18:3 *trans*-9, *cis*-12, *trans*-15; C18:3 *cis*-9, *cis*-12, *trans*-15; C18:3 *cis*-9, *trans*-12, *cis*-15; and C18:3 *trans*-9, *cis*-12, *cis*-15).

Total TFA.—Sum of C18:1 TFA, C18:2 TFA, and C18:3 TFA.

G. Calculations

(*1*) *Calculation of response factors.*—Determine the area of the peaks attributable to each FAME present in the calibration standard mixture, **D**(**p**), and calculate Rf_i, their respective response factors relative to the internal standard (C11:0):

$$Rf_i = \frac{m'_i \cdot A'_O}{m'_O \cdot A'_i}$$

where m'_i = mass fraction of $FAME_i$ in the calibration standard solution, **D**(**p**); A'_O = peak area of C11:0 in the calibration standard solution chromatogram; m'_O = mass of C11:0 in the calibration standard solution, **D**(**p**); and A'_i = peak area of $FAME_i$ in the calibration standard solution chromatogram.

The variation between three injections is optimal when coefficients of variation are <2.5.

Note: The response factors calculated for C18:2 *n*-6 *cis* could be applied for the quantification of C18:2 CLAs, and those calculated for C18:3 *n*-3 *cis* could be applied for C18:3 *trans* isomers.

(*2*) *Fatty acids expressed on the product.*—Calculate the mass fraction of the individual fatty acid components (FA_i), expressed in grams FA_i per 100 g product in the test sample by using the following equation:

$$gFA_i/100g\ \ product = \frac{m_o \cdot A_i \cdot Rf_i \cdot S_i(FA) \cdot 100}{A_o \cdot m}$$

where m_O = mass in milligrams of C11:0 internal standard added to the sample solution **D**(**j**); A_i = peak area of $FAME_i$ in the sample chromatogram; Rf_i = response factor, calculated according to **G**(*1*); $S_i(FA)$ = stoichiometric factor to convert $FAME_i$ to FA_i (Table **2012.13**); A_O = peak area of C11:0 internal standard in the sample chromatogram; and m = mass in milligrams of the test portion.

Note: For powder samples, the result is expressed in grams FA_i per 100 g product, which can be converted to reconstituted liquid product (i.e., 25 g powder dissolved in 200 g water).

Note: In the case of fatty acids analysis carried out on fat extracted from foods, the mass of test portion m corresponds to fat and not to the finished product. Consequently, fatty acids results are expressed in grams fatty acids per 100 g fat, which can then be converted into grams fatty acids per 100 g product, with the fat extraction value determined with an appropriate validated extraction method.

(*3*) *Fatty acids expressed on the total fat.*—Calculate the mass fraction of the individual components expressed in grams FA_i per 100 g fat in the test sample by using the following equation:

$$gFA_i/100g\ \ fat = \frac{gFA_i/100g\ \ product \cdot 100}{\%Fat}$$

This calculation can be performed only when the fat content is determined with an appropriate and validated fat extraction method. Do not use the declared fat value for the expression of fatty acids on finished products due to possible imprecision between fat labeled and fat extracted.

(*4*) *Sum of class or group of fatty acids.*—Calculate the mass fraction of all fatty acids included in a group or in a class of fatty acids by the simple addition of individual fatty acids results corresponding to each class or group (expressed in grams fatty acids (FA) per 100 g product):

$$\sum FA = (gFA_{i1}/100g + gFA_{i2}/100g + gFA_{i3}/100g)$$

Table 2012.13. Stoichiometric factors for converting FAMEs to fatty acids

Chain length	Position of unsaturation (terminal methyl carbon)	Configuration	Group	Abbrev.	FAME mol. wt, g/mol	FA mol. wt, g/mol	TAG mol. wt, g/mol	$S_i(FA)$[a]
C4:0			SFA		102.1	88.1	302.4	0.863
C6:0			SFA		130.2	116.2	386.5	0.892
C8:0			SFA		158.3	144.2	470.7	0.911
C10:0			SFA		186.3	172.3	554.9	0.925
C12:0			SFA		214.4	200.3	639.0	0.935
C14:0			SFA		242.4	228.4	723.2	0.942
C14:1	ω-5 (or *n*-5)	*Cis*	MUFA[b]		240.4	226.4	717.1	0.942
C15:0			SFA		256.4	242.4	765.3	0.945
C15:1	ω-5 (or *n*-5)	*Cis*	MUFA		254.4	240.4	759.2	0.945
C16:0			SFA		270.5	256.4	807.3	0.948
C16:1	ω-7 (or *n*-7)	*Cis*	MUFA		268.5	254.4	801.3	0.948
C17:0			SFA		284.5	270.5	849.4	0.951
C17:1	ω-7 (or *n*-7)	*Cis*	MUFA		282.5	268.4	843.4	0.950
C18:0			SFA		298.5	284.5	891.5	0.953
C18:1 TFA		*Trans*[c]			296.5	282.5	885.5	0.953
C18:1	ω-9 (or *n*-9)	*Cis*	MUFA		296.5	282.5	885.5	0.953
C18.2 TFA		*Trans*[c]			294.5	280.5	879.4	0.952
C18:2	ω-6 (or *n*-6)	*Cis*	PUFA	LA[d]	294.5	280.5	879.4	0.952
C18:2 CLA	ω-6 (or *n*-6)	*Cis/trans*	PUFA	CLA	294.5	280.5	879.4	0.952
C18:3	ω-6 (or *n*-6)	*Cis*	PUFA		292.5	278.4	873.4	0.952
C18:3 TFA		*Trans*[c]			292.5	278.4	873.4	0.952
C18:3	ω-3 (or *n*-3)	*Cis*	PUFA	ALA[e]	292.5	278.4	873.4	0.952
C20:0			SFA		326.6	312.5	975.7	0.957
C20:1	ω-9 (or *n*-9)	*Cis*	MUFA		324.6	310.5	969.6	0.957
C20:2	ω-6 (or *n*-6)	*Cis*	PUFA		322.5	308.5	963.6	0.957
C20:3	ω-6 (or *n*-6)	*Cis*	PUFA		320.5	306.5	957.5	0.956
C20:3	ω-3 (or *n*-3)	*Cis*	PUFA		320.5	306.5	957.5	0.956
C20:4	ω-6 (or *n*-6)	*Cis*	PUFA	ARA[f]	318.5	304.5	951.5	0.956
C20:5	ω-3 (or *n*-3)	*Cis*	PUFA	EPA[g]	316.5	302.5	945.4	0.956
C22:0			SFA		354.6	340.6	1059.9	0.960
C22:1	ω-9 (or *n*-9)	*Cis*	MUFA		352.6	338.6	1053.8	0.960
C22:2	ω-6 (or *n*-6)	*Cis*	PUFA		350.6	336.6	1047.8	0.960
C22:6	ω-3 (or *n*-3)	*Cis*	PUFA	DHA[h]	342.5	328.5	1023.6	0.959
C24:0			SFA		382.7	368.7	1144.0	0.963
C24:1	ω-9 (or *n*-9)	*Cis*	MUFA		380.7	366.6	1137.9	0.963

[a] $S_i(FA)$ = Stoichiometric factor to convert $FAME_i$ to FAi.

[b] MUFA = Monounsaturated fatty acid.

[c] Does not include TFAs in MUFA and PUFA sums.

[d] LA = Linoleic acid.

[e] ALA = α-Linolenic acid.

[f] ARA = Arachidonic acid.

[g] EPA = Ecosapentaenoic acid.

[h] DHA = Docosahexaenoic acid.

(*5*) *Performance of the transesterification.*—Record the areas of the two internal standard peaks (methyl undecanoate and tritridecanoin) in the analyzed samples. The performance of transesterification (Pt) expressed as a percentage, is calculated on the recovery of the tritridecanoin as a second internal standard as follows:

$$Pt = \frac{m_{c11} \times A_{c13} \times R_{c13} \times S_{c13}(TAG)}{A_{c11} \times m_{c13}} \times 100$$

where m_{c11} is the mass in milligrams of the C11:0 internal standard added to the solution; A_{c13} is the peak area of the C13:0 internal standard in the chromatogram; R_{c13} is the response factor of C13:0 relative to C11:0, calculated according to **G**(*1*); S_{c13} is the stoichiometric factor to convert C13:0 FAME into C13:0 TAG; A_{c11} is the peak area of the C11:0 internal standard in the chromatogram; and m_{c13} is the mass in milligrams of the C13:0 internal standard added to the solution.

The performance of the transesterification method should be always 100.0±2.0%. When the performance of the transesterification is >102.0 or <98.0%, the origin of the problem could be the following: incomplete transesterification, partial degradation of internal standard(s), or a matrix effect problem. The evaluation of transesterification performance in a blank sample can be used to monitor the stability of reagents and chemicals.

Results and Discussion

Forty-six analytes (Table 2) were recorded in 12 selected samples (Table 1) analyzed as double blinds (i.e., 24 analyses) by 18 participants; in total, 19 872 results were collected from the study. The results were carefully evaluated and summarized in an Excel template used for statistical evaluation. Single values reported for one double-blind sample were removed from the evaluation, and a list of fatty acids was selected according to the composition (dairy versus nondairy) of analyzed samples and their abundance. Outlier values were removed based on Cochran and/or Grubbs tests following the ISO 5725 guideline. All statistical decisions regarding the evaluation of data were carefully recorded and provided to the ERP to assist in their decision to accord Final Action status in July 2014.

Results corresponding to Samples 1–6 were expressed in grams per 100 g finished product, except for Sample 5 (cheese), which are expressed in grams per 100 g extracted fat from cheese. Results corresponding to SPIFAN Samples 7–9 in powder form were expressed in grams per 100 g reconstituted product (25 g powder with 200 g water), and Samples 10–12 were expressed in grams per 100 g liquid products. Results for Samples 7–12 were reported using criteria defined in AOAC SMPR 2012.011.

As previously discussed with the ERP during SLV data evaluation, the requirements for repeatability and reproducibility are not fully consistent with the whole range of fatty acids concentration found in the samples. Values were given for fatty acid concentrations of <0.5, ≥0.5 to <3.0, and ≥3.0 g/100 g; however, the quantification limit is (≤0.001) is 500 times lower than the lower fatty acid concentration indicated in SMPR (0.5). The performance requirement at the level of 0.5 cannot be the same for a fatty acid in a concentration at 0.499 in a sample and at 0.001 in another sample (500 times lower). As a consequence, additional limits for repeatability and reproducibility values also need to be fixed in the SMPR for concentrations below 0.5. Proposed repeatability and reproducibility limits are shown in Table 3.

The transesterification performance (i.e., recovery between C11:0 FAME and C13:0 TAG) was monitored in all samples and ranged between 98.9 and 100.0% with an RSD value between 0.9 and 1.6%, except for a cheese sample (2.7%).

A questionnaire was sent to all participants, along with an invitation to give comments about the performance of the method in their laboratory. Feedback was requested with respect

Table 2. Analytes

Fatty acid	Abrev.	Fatty acids analyzed
Individual fatty acid		
C18:2 *n*-6	LA	C18:2 *n*-6
C18:3 *n*-3	ALA	C18:3 *n*-3
C20:4 *n*-6	ARA	C20:4 *n*-6
C20:5 *n*-3	EPA	C20:5 *n*-3
C22:6 *n*-3	DHA	C22:6 *n*-3
Group of fatty acid		
Saturated fatty acids	SFAs	C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C22:0, and C24:0
Monounsaturated fatty acids	MUFAs	C14:1 *n*-5, C15:1 *n*-5, C16:1 *n*-7, C17:1 *n*-7, C18:1 *n*-9 (and other *cis* isomers), C20:1 *n*-9, C22:1 *n*-9, and C24:1 *n*-9
Polyunsaturated fatty acids	PUFAs	C18:2 *n*-6, C18:3 *n*-6, C18:3 *n*-3, C20:2 *n*-6, C20:3 *n*-3, C20:3 *n*-6, C20:4 *n*-6, C20:5 *n*-3, C22:2 *n*-6, and C22:6 *n*-3
Trans fatty acids	TFAs	C18:1 *trans*, C18:2 *trans*, and C18:3 *trans*
Omega-3	ω-3	C18:3 *n*-3, C20:3 *n*-3, C20:5 *n*-3, and C22:6 *n*-3
Omega-6	ω-6	C18:2 *n*-6, C18:3 *n*-6, C20:2 *n*-6, C20:3 *n*-6, C20:4 *n*-6, and C22:2 *n*-6
Omega-9	ω-9	C18:1 *n*-9, C20:1 *n*-9, C22:1 *n*-9, and C24:1 *n*-9
Sum of all fatty acids		
Total fatty acids		All fatty acids (including CLAs and omega fatty acids)
Transesterification performance	TP	C11:0 and C13:0 (internal standards)

to four areas: (*1*) remarks regarding the collaborative study's organization (i.e., information, sample, schedule); (*2*) comments about the procedure used for sample analysis; (*3*) statements about insufficient information provided in the method (or inconsistency); and (*4*) remarks about the method not being well implemented in laboratory. In general, all comments were positive with respect to the use of this complex chromatographic method in routine analysis, which necessitates an experienced and trained analyst. All comments were summarized and sent to the ERP for review in July 2014 prior to receiving Final Action status.

The method has demonstrated its compliance with the applicability statement of AOAC SMPR 2012.011 and has been shown, in this collaborative study, to be suitable for the analysis of fatty acids in selected food matrixes. The majority of results provided for individual and groups of fatty acids were in agreement with expectations (i.e., results gained with proficiency tests and SLV).

Nevertheless, this kind of analytical method requires particular attention for the chromatography part, which is the source of principal differences observed in the results (i.e., response factors of the instrument, coelution, and wrong peak identification and integration, but also errors in the reporting). The accurate identification and quantification of each peak corresponding to *trans* isomers is very important because they can significantly impact the TFA sums. The C18:3 *trans* isomers (having possibly two, three, or four different peaks corresponding to *trans* isomers) are the most difficult category of isomers to quantify in food matrixes due to possible coelution with other fatty acids.

The global performance of the method is satisfactory because RSD_r and RSD_R values for labeled fatty acids were below 85% of limits fixed in the SMPR for all concentrations. RSD_R values were summarized separately for labeled fatty acids in SPIFAN materials and ISO-IDF materials due to different expression of results (Table 4). Results compared to the SMPR values are shown in Table 5.

Table 3. Proposed limits for repeatability and reproducibility values

Concentration, g/100 g	Repeatability (RSD_r)	Reproducibility (RSD_R)
<0.05 and ≥0.005	10	25
<0.005 and ≤0.001	15	40

Conclusions

A multilaboratory collaborative study of AOAC First Action Method **2012.13** "Determination of Labeled Fatty Acids Content in Milk Products and Infant Formula (and Adult/Pediatric Nutritional Formula) by Capillary Gas Chromatography" and ISO 16958:2015 | IDF 231:2015 was done. This method was applied to representative dairy, infant formula, and adult/pediatric nutritional formula products and demonstrated acceptable reproducibility precision for all fatty acids (i.e., 46 individuals and/or groups) analyzed for these categories of products.

Recommendations

A detailed report summarizing the outcomes of this collaborative study was submitted with the recommendation that AOAC First Action Method **2012.13** be accepted as a SPIFAN-endorsed AOAC Final Action Method. The AOAC ERP evaluated the collaborative study data in September 2014 and endorsed the recommendation, which was subsequently approved by the Official Methods Board in October 2014.

Table 4. Results of the collaborative study

	SPIFAN materials[a]			ISO-IDF materials[b]		
		RSD_R, %			RSD_R, %	
Fatty acid	Range	Min.	Max.	Range	Min.	Max.
TFA (total)	0.006–0.027	21.31	42.47	0.008–5.056	8.69	32.92
SFAs	0.195–1.945	1.92	6.50	0.812–57.777	2.38	5.72
PUFAs	0.324–1.129	4.58	8.86	0.107–2.795	2.73	11.17
MUFAs[c]	0.803–4.552	4.14	8.64	0.717–18.894	4.25	8.80
Omega-3 (ω-3)	0.055–0.121	5.32	8.40	0.022–0.637	4.47	11.68
Omega-6 (ω-6)	0.268–1.019	4.61	8.96	0.051–1.262	2.86	7.80
Omega-9 (ω-9)	0.799–4.543	4.14	8.64	0.631–16.538	4.40	9.04
C18:2 *n*-6 (LA)[d]	0.267–1.017	4.28	8.48	0.044–1.036	2.83	11.81
C18:3 *n*-3 (ALA)[e]	0.048–0.121	4.86	7.68	0.02–0.574	4.90	9.53
C20:4 *n*-6 (ARA)[f]	0.016–0.023	3.61	7.34	0.003–0.089	10.65	33.71
C22:6 *n*-3 (DHA)[g]	0.008–0.011	5.47	14.64	0.006	8.47	

[a] Results expressed in grams per 100 g reconstituted product for powder (25 g + 200 g water) and in grams per 100 g for liquid.

[b] Results expressed in grams per 100 g product (powder and liquid).

[c] MUFAs = Monounsaturated fatty acids.

[d] LA = Linoleic acid.

[e] ALA = α-Linolenic acid.

[f] ARA = Arachidonic acid.

[g] DHA = Docosahexaenoic acid.

Table 5. Method performance versus SMPR 2012.011

Parameter	SLV	MLT[a]	SMPR 2012.011[b]
Matrixes	SLV test Matrixes kit (12 samples)	SLV test Matrixes kit (6 samples)[c]	All forms of infant, adult, and/or pediatric formula (powders, ready-to-feed liquids, and liquid concentrates)
LOQ, g/100 g	0.001[d]	0.001[d]	≤0.001[d]
Analytical range, g/100 g	0.001–7.94[d]		≤0.001–8.00[d]
Recovery	100.0–102.9% (for C18:2 *n*-6 and C18:3 *n*-3)		90–110% for labeled fatty acids; 80–110% of mean spiked recovery over the range of the assay
Repeatability (RSD_r), %			
≥3.0[d]	<1.3	2.5	≤2.0
≥0.5 to <3.0[d]	<1.7	1.2–4.8	≤4.0
≥0.05 to <0.5[d]	<1.8	1.9–7.0	≤7.0
≥0.005 to <0.05[d]	<3.5	2.1–(16.6)[e]	<10
≤0.001 to <0.005[d]	<6.2		<15
Intralaboratory precision (RSD), %			
≥3.0[d]	<1.1		Not specified
≥0.5 to <3.0[d]	<1.7		
≥0.05 to <0.5[d]	<2.9		
≥0.005 to <0.05[d]	<6.3		
≤0.001 to <0.005[d]	<7.0		
Reproducibility (RSD_R), %			
≥3.0[d]	Not done	5	≤4
≥0.5 to <3.0[d]		1.9–9.0	≤8
≥0.05 to <0.5[d]		4.9–8.4	≤15
≥0.005 to <0.05[d]		3.6–(42.54)[e]	<25
≤0.001 to <0.005[d]			<40

[a] MLT = Multilaboratory testing.

[b] SMPR 2012.011, Final, September 29, 2012. 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.

[c] The table is valid only for SPIFAN Samples 7 to 12.

[d] Grams of fatty acids per 100 g reconstituted final product.

[e] Results in parentheses correspond to TFA amounts found in SPIFAN materials in very low amounts.

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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*℠ 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*℠ **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.

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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.
Corresponding author's e-mail: richard.zywicki@covance.com
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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.

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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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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.

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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.

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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

Requirements (SMPR 2012.009)	Mean, mg/100 g	≤5% RSD_r, %[a]	≤15% 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

Requirements (SMPR 2012.009)	n[a]	Mean, mg/100 g	≤5% RSD_r, %	≤15% 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.

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INFANT FORMULA AND ADULT NUTRITIONALS

Vitamin C in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography with UV Detection: Collaborative Study, Final Action 2012.22

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To determine the repeatability and reproducibility values of the AOAC INTERNATIONAL First Action Method 2012.22, *Vitamin C in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography with UV Detection*, a collaborative study was organized. The study was divided into two parts: method setup and qualification of participants (part 1) and collaborative study participation (part 2). During part 1, each 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, where they analyzed 10 samples in blind duplicates. Two of the samples were suspected of spoilage during the test and new cans of the same type of product were analyzed by a subset of laboratories in part 3. The results were compared with *Standard Method Performance Requirement* (SMPR®) 2012.012 established for vitamin C. The precision results were within the requirements stated in the SMPR: 1.4–7.3% and 3.2–11.4% respectively, for repeatability and reproducibility. Finally, Horwitz ratio values were all <2 (0.5–1.7). The Expert Review Panel for Stakeholder Panel for Infant Formula and Adult Nutritionals Nutrient Methods determined that the data presented met the SMPR and therefore recommended the method be granted Final Action status.

Vitamin C (L-ascorbic acid) plays an important role in oxidative stress reactions and is involved in a number of metabolic functions (1). Because humans are unable to synthetize vitamin C, its supply must be ensured through adequate dietary intake. Daily requirements can vary from 75 mg in adult women to 90 mg in adult men (2), and Codex has established a lower limit of 10 mg/100 kcal and a guidance upper level of 70 mg/100 kcal in infant formula and foods for special medical purposes (3). Several official AOAC INTERNATIONAL Methods exist for the analysis of vitamin C in foods, and in particular, in infant formula, as reviewed elsewhere (4). Fontannaz et al. (5) published a method for the quantification of ascorbic and isoascorbic acid in fortified foods, including infant formula and nutritional products. A modification of this method was proposed to the Expert Review Panel for Stakeholder Panel for Infant Formula and Adult Nutritionals Nutrient Methods (SPIFAN) and was approved as AOAC First Action Method **2012.22** in 2012 (6), with a recommendation to advance to a multilaboratory collaborative study. This paper presents the results of the collaborative study.

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The method was approved by the AOAC Official Methods Board as Final Action. *See* "Standards News," (2016) *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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Samples

The study took place using SPIFAN matrixes, which represent most of the products in the scope of the project (infant formula and adult nutritionals made from any combination of milk, soy, rice, whey, hydrolyzed protein, starch, and amino acids, with and without intact protein). All samples were blinded and codified before being sent to participating laboratories.

AOAC Official Method 2012.22
Vitamin C in Infant Formula and Adult/Pediatric Nutritional Formula
Liquid Chromatography with UV Detection
First Action 2012
Final Action 2016

[Applicable to the determination of vitamin C (L-ascorbic acid) in infant formula and adult/pediatric nutritional formula by LC–UV.]

Caution: Refer to Material Safety Data Sheets prior to use of chemicals. Use appropriate personal protective equipment when performing tests.

A. Principle

L-Ascorbic acid is extracted from the sample using trichloroacetic acid (TCA) in the presence of tris [2-carboxyethyl] phosphine (TCEP) as a reducing agent. Ascorbic acid is then determined by ultra-HPLC (UHPLC) or HPLC with UV detection at 265 nm. Separation takes place in a C_{18} column

using decylamine as an ion-pairing agent in a sodium acetate buffer solution (pH 5.4) containing TCEP.

B. Apparatus

(a) *Balances.*—With readability of 0.1 mg and 0.01 g.

(b) *pH meter.*—Metrohm 691 (Herisau, Switzerland), or equivalent.

(c) *Folded paper filters.*—Grade 597 ½.

(d) *Syringe filters.*—0.22 or 0.45 µm pore size.

(e) *Chromatographic system.*—HPLC or UHPLC system equipped with a quaternary or binary pump, a sample injector, a UV-Vis detector (or optionally, a photodiode array detector), a degassing system, and data software.

(f) *UHPLC column.*—Waters Acquity UPLC ethylene bridged hybrid C_{18} column (1.75 µm, 2.1 × 100 mm; or equivalent).

(g) *HPLC column.*—LiChrospher RP-18 column (5 µm, 250 × 4.6 mm; or equivalent).

C. Reagents and Standards

(a) *Acetonitrile.*—HPLC grade, Merck (Geneva, Switzerland); or equivalent.

(b) *Ascorbic acid.*—>99%, Fluka (Buchs, Switzerland), or equivalent.

(c) *Decylamine.*—Fluka, or equivalent.

(d) *Phosphoric acid.*—85%, Merck; or equivalent.

(e) *Ultrapure water.*—Resistivity >18 MΩ/cm.

(f) *Sodium acetate trihydrate.*— Merck, or equivalent.

(g) *TCA.*—Merck, or equivalent.

(h) *TCEP.*—Fluka, or equivalent.

(i) *Isoascorbic acid.*—Fluka, or equivalent.

(j) *Orotic acid.*—Sigma, or equivalent.

D. Preparation of Solutions

(a) *Sodium acetate solution (500 mmol/L, pH 5.4).*—In a 500 mL volumetric flask, weigh 34.0 g sodium acetate trihydrate and add ~400 mL water for dissolution. Adjust the pH to 5.4 with phosphoric acid (85%) and dilute to volume with water.

(b) *TCA (15%).*—In a 500 mL volumetric flask, weigh 75.0 g TCA, dissolve the compound, and dilute to volume with water.

(c) *TCEP (250 µg/mL).*—In a 500 mL volumetric flask, weigh 125 mg TCEP, dissolve the compound, and dilute to volume with water.

(d) *Mobile phase for UHPLC.*—In a 250 mL flask, mix 0.4 g decylamine, 2.5 mL acetonitrile, 25 mL sodium acetate solution 500 mmol/L (pH 5.4), and 205 mL water (do not dilute to volume). Adjust the pH to 5.4 with phosphoric acid (85%). Add 10 mg TCEP.

(e) *Mobile phase for HPLC.*—In a 1000 mL flask, mix 1.6 g decylamine, 80 mL acetonitrile, 100 mL sodium acetate solution 500 mmol/L (pH 5.4), and 820 mL water (do not dilute to volume). Adjust the pH to 5.4 with phosphoric acid (85%). Add 50 mg TCEP.

E. Preparation of Standards

Note: Vitamin C is sensitive to light and oxygen. Conduct operations under subdued light conditions, or use amber glassware. Keep all solutions away from direct light.

(a) *Ascorbic acid stock solution (500 µg/mL).*—In a 25 mL amber glass volumetric flask, weigh 12.5 mg ascorbic acid. Dissolve and dilute to volume with TCEP solution. This solution can be kept for 3 months if stored at 4°C away from light.

(b) *Ascorbic acid intermediate standard solution (50 µg/mL).*— In a 10 mL amber glass volumetric flask, pipet 1 mL stock solution. Dilute to volume with TCEP solution. This solution can be used for 1 month if stored at 4°C away from light.

(c) *Ascorbic acid calibration standard solutions (0.5, 1.0, 2.0, 3.0, 5.0, 7.5, and 10.0 µg/mL).*—In 10 mL amber glass volumetric flasks, pipet 0.1, 0.2, 0.4, 0.6, 1.0, 1.5, and 2.0 mL intermediate standard solution. Dilute to volume with mobile phase to prepare the respective concentrations given above.

F. Sample Preparation

(a) *Reconstitution of powder samples.*—(*1*) Weigh 25 g powder in a 250 mL brown glass beaker and add 10 mg TCEP.

(*2*) Add 200 g warm water (40°C). Mix well until dissolution is complete.

Proceed to **(b)** as soon as possible as vitamin C can degrade rapidly. Do not let the reconstituted samples stand for >30 min.

(b) *Extraction.*—(*1*) Weigh 2 g liquid or reconstituted sample in a 10 mL amber glass volumetric flask.

(*2*) Add 4 mL TCEP solution and 2 mL TCA (15%).

(*3*) Dilute to volume with water.

(*4*) Filter the solution through a folded paper filter.

(*5*) Transfer 1 mL filtrate to a 10 mL amber glass volumetric flask containing 1 mL acetate solution (pH 5.4) and dilute to volume with mobile phase.

(*6*) Filter ~2 mL through a 0.22 or 0.45 µm membrane into an HPLC vial.

(*7*) Proceed to chromatographic analysis using either UHPLC conditions in **G(a)** or HPLC conditions in **G(b)**.

G. Analysis

(a) *UHPLC conditions.*—(*1*) Injection volume.—5 µL.

(*2*) *Autosampler temperature.*—10°C.

(*3*) *Column temperature.*—25°C.

(*4*) *Flow rate.*—0.35 mL/min.

(*5*) *Run time.*—4.0 min.

(*6*) *Mobile phase for UHPLC.*—*See* **D(d)**: 0.4 decylamin, 2.5 mL acetonitrile, 25 mL sodium acetate 500 mmol/L (pH 5.4), 205 mL water, and 10 mg TCEP (pH 5.4).

(*7*) *Detection wavelength.*—265 nm.

Note: At the end of each analytical series, rinse the column with acetonitrile–water (1 + 1, v/v) for 10 min at 0.4 mL/min.

(b) *HPLC conditions.*—(*1*) Injection volume.—25 µL.

(*2*) *Autosampler temperature.*—10°C.

(*3*) *Column temperature.*—25°C.

(*4*) *Flow rate.*—1.0 mL/min.

(*5*) *Run time.*—20 min.

(*6*) *Mobile phase for HPLC.*—*See* **D(e)**: 1.6 g decylamine, 80 mL acetonitrile, 100 mL sodium acetate 500 mmol/L (pH 5.4), 820 mL water, and 50 mg TCEP (pH 5.4).

(*7*) *Detection wavelength.*—265 nm.

Note: At the end of each analytical series, rinse the column with acetonitrile–water (1 + 1, v/v) for 60 min at 1.0 mL/min.

(c) *System suitability test*.—Equilibrate the chromatographic system for ≥0.5 h. Inject a working standard solution of ascorbic acid at least six times and check the peak retention times and response (peak height or area). Ensure orotic acid and isoascorbic acid are fully resolved from ascorbic acid by injecting separate standard solutions of each compounds (prepared as stated for ascorbic acid). If the acids are not resolved, decrease the pH of the mobile phase to 5.0 or increase the amount of acetonitrile used.

(d) *Calibration*.—Perform single injections of working standard solutions, as a minimum at the beginning and end of each analytical series. Establish the calibration curve (seven points) by plotting peak response (height or area) vs ascorbic acid concentration, perform linear regression, and calculate the slope and intercept of the calibration curve.

(e) *Analysis*.—Perform single injections of sample solutions.

(f) *Identification*.—Identify the ascorbic acid peak in the chromatograms of the sample solutions by comparing it with the retention time and UV spectrum of the corresponding peak in the standard solution.

H. Calculations

Calculate the concentration of vitamin C in mg ascorbic acid/100 g expressed in as-is ready-to-feed (RTF) products—or as reconstituted powder for powder samples—as follows:

$$C = \frac{(A - I) \times V_1 \times V_3 \times 100}{S \times m \times V_2 \times 1000}$$

where A is the response (height or area) of the ascorbic acid peak obtained for the sample solution, I is the intercept of the calibration curve, S is the slope of the calibration curve, m is the weight of the test portion in grams (2.0 g), V_1 is the volume of the test solution (volume used to dissolve the test portion) in milliliters (10 mL), V_2 is the volume used in the sample dilution (1.0 mL), and V_3 is the volume of the final sample dilution (10 mL).

Note: If results expressed in the powder sample are needed, use the reconstitution rate for the calculation C × (225/25).

I. Collaborative Study Protocol

Part 1.—All participant laboratories received two practice samples and were asked to analyze each of them in duplicate (two extractions from each reconstituted sample). Any deviation from the written method was to be recorded and reported. Results were communicated to the Study Director using the electronic template provided with the protocol. The participants were asked to report final vitamin C results, peak responses for standard curves and samples, and the different masses used during sample preparation. After review by the Study Director, results within a range of expected levels (average ±2 × S_R) were used to identify the laboratories that qualified for part 2 of the study.

Part 2.—All qualified laboratories received a second shipment containing 20 coded products, corresponding to 10 products in blind duplicates. The samples were a set of infant formula and adult nutritional products, representing a wide range of commercially available products. The laboratories were asked to analyze all the samples (a single extraction from each liquid or reconstituted powder) on 2 days (10 samples/day). Each sample was assigned to either days 1 or 2. The blind duplicates were assigned for analysis on the same day. Results were communicated to the Study Director using an electronic template similar to the one used in part 1.

Part 3.—Two of the samples [infant formula RTF (milk-based) and adult nutritional powder (low-fat)] failed to meet acceptance criteria during part 2. Due to the questionable integrity of the samples (some laboratories reported product spoilage) and the fact that the samples had reached their expiration date, new products representing the same type of matrix were sent to a subset of 12 laboratories for analysis following the same protocol. Results were transmitted to the Study Director using the same electronic template as in parts 1 and 2.

J. Statistical Evaluation

After data collection, outliers were detected using Cochran's and Grubbs' tests. Average concentrations, S_r, and RSD_r were estimated from the blind duplicates. S_R, RSD_R, and Horwitz ratio (HorRat) values (i.e., RSD_R/predicted RSD_R) were also estimated. Details on statistical analysis can be found in *Official Methods of Analysis*SM "*Appendix D*: *Guidelines for Collaborative Study Procedures To Validate Characteristics of a Method of Analysis*" (7).

Results and Discussion

Part 1

Twenty-six laboratories initially agreed to participate in the collaborative study. Two laboratories dropped out during part 1 as a result of issues related to the availability of resources. The remaining 24 laboratories set up the method as described in the protocol. Fourteen laboratories used the UHPLC conditions as described in the protocol, whereas the remaining 10 used previously published HPLC conditions (5). No differences could be found between the provided results in either condition, and thus evaluation was performed combining all results.

During method setup, it was brought to the attention of the Study Director that there was a need to establish suitability testing to ensure proper chromatographic separation between ascorbic, isoascorbic, and orotic acids. This suitability testing was added to the method as presented in this paper.

One laboratory did not receive the practice samples due to customs restrictions. The laboratory qualified for part 2 by using results from the reference samples. After data compilation, average concentrations and S_r and S_R were calculated. Another laboratory reported single results and was not included in the statistical evaluation, although it qualified for part 2. Two laboratories reporting data above or below the average (±2 × SD) were flagged as possible outliers and informed accordingly. Nevertheless, the two laboratories were accepted to continue because their results, although questionable, were still within ±3 × SD.

Part 2

Of the 24 laboratories providing results for practice samples and qualified to continue to part 2, two did not receive the full collaborative study set due to customs restrictions. The

remaining 22 laboratories reported valid data. The full set of original data is listed in Table 1, whereas the results of the statistical evaluation are presented in Table 2. No significant differences could be observed between laboratories using HPLC (5) or UHPLC (6) conditions, when suitability conditions were respected (i.e., the separation of orotic acid and isoascorbic acid from ascorbic acid). In general, the precision results (repeatability and reproducibility) were well within the limits stated in *Standard Method Performance Requirement* (SMPR®) 2012.012 (8). Two samples showed much higher variability (RSD>25%) than the SMPR. By excluding these values from the statistical evaluation, repeatability ranged from 1.4 to 7.3% and reproducibility from 3.2 to 11.4%. HorRat values were all <2 (0.4–1.7).

These results were submitted to the AOAC Expert Review Panel (ERP) in January 2015. Based on the aforementioned data, the method was not recommended to move forward to Final Action status because the high repeatability and reproducibility values had been obtained in two samples [infant formula RTF (milk-based) and adult nutritional powder (low-fat)].

Part 3

The ERP offered the method authors the opportunity to test fresh samples from the new SPIFAN kit. Additional multi-laboratory testing (MLT) on these two matrixes was therefore organized (part 3).

Twelve laboratories agreed to participate in this study. Two did not receive samples due to customs restrictions. The remaining 10 laboratories reported valid data, which are presented in Table 3.

After statistical analysis (*see* Table 4), the results for the adult nutritional powder (low-fat) product were well within

Table 1. Full set of original data[a]

Lab No.	Adult nutritional RTF (high-fat)[b]		SRM 1849a[c]		Child formula powder		Adult nutritional powder (milk protein-based)		Infant formula powder (soy-based)		Infant formula RTF (milk-based)		Adult nutritional powder (low-fat)		Adult nutritional RTF (high-protein)		Infant elemental powder		Infant formula powder (part. hyd., soy-based)[d]	
1	14.3	13.7	8.3	8.2	5.1	2.5	3.0	6.3	4.0	10.4	2.5	3.0	12.6	5.1	21.8	21.4	32.8	28.0	19.5	20.4
2	16.8	15.9	8.1	8.0	4.9	3.7	4.5	6.2	9.8	9.9	3.7	4.5	9.1	5.3	20.2	19.9	28.4	31.4	19.7	19.6
3	16.5	15.9	7.8	8.0	4.7	2.3	2.3	6.3	9.7	10.1	2.3	2.3	12.4	11.2	18.7	18.8	31.9	27.7	19.5	19.0
4	17.3	16.0	7.9	7.7	4.6	3.1	3.1	5.9	10.1	10.0	3.1	3.1	8.5	13.3	19.8	20.5	35.8	35.6	18.5	18.8
5	19.0	17.2	8.2	8.0	5.1	4.5	3.1	6.3	10.3	10.3	4.5	3.1	8.6	13.0	19.9	19.1	28.7	33.7	19.1	19.4
6	18.8	19.1	8.5	7.5	4.9	4.9	4.0	6.2	9.6	9.9	4.9	4.0	15.8	7.8	20.1	20.1	36.0	42.4	19.5	20.0
7	17.8	17.4	7.9	8.2	4.9	6.2	4.0	6.4	10.0	9.9	6.2	4.0	12.9	4.8	19.8	19.6	30.6	35.2	19.0	19.4
8	19.8	19.6	9.5	9.3	5.0	2.4	4.3	6.7	10.3	6.8	2.4	4.3	9.0	13.2	20.9	20.2	32.9	32.8	16.2	17.8
9	17.5	19.5	7.9	8.7	4.8	3.4	3.3	6.5	11.0	11.1	3.4	3.3	14.9	14.7	16.5	20.7	36.3	40.4	21.7	21.1
10	18.6	19.1	8.3	8.1	4.8	2.5	3.0	6.6	10.8	10.2	2.5	3.0	13.4	13.3	20.9	20.1	36.0	35.5	19.7	19.5
11	19.5	19.3	9.0	9.3	5.2	5.1	3.3	7.1	11.7	11.8	5.1	3.3	14.3	14.5	21.4	20.7	37.7	34.4	20.6	20.4
12	12.8	12.8	8.4	8.1	4.5	2.5	2.4	6.0	10.1	10.0	2.5	2.4	4.9	4.9	15.9	15.7	27.1	32.8	18.3	18.5
13	16.8	16.6	7.9	7.7	5.1	5.4	2.7	6.4	10.5	10.4	5.4	2.7	NA[e]	NA	18.3	18.4	NA	NA	19.2	19.7
14	15.1	16.7	7.8	7.7	4.8	3.5	1.8	6.4	9.2	9.1	3.5	1.8	14.9	15.9	16.3	16.8	32.1	33.7	17.8	18.8
15	20.4	21.4	8.3	8.5	5.3	7.2	5.1	7.4	11.7	10.2	7.2	5.1	14.8	11.9	24.0	23.2	41.1	40.6	22.3	24.0
16	19.4	18.4	8.5	8.2	5.1	2.9	4.5	6.7	10.9	11.0	2.9	4.5	13.8	5.8	20.4	20.3	36.8	32.0	20.7	20.6
17	NA	NA	8.7	8.0	3.8	3.6	NA	6.1	7.8	8.4	NA	NA	12.9	13.0	NA	NA	37.6	36.2	15.9	16.4
18	19.1	19.5	8.5	8.4	5.3	3.0	2.9	6.0	10.7	10.5	3.0	2.9	9.3	14.0	21.9	18.9	36.9	37.5	21.4	21.2
19	17.2	18.4	6.9	7.7	4.9	4.5	3.6	6.4	10.3	10.7	4.5	3.6	12.9	3.1	20.5	18.2	34.1	31.7	20.1	19.9
20	19.1	18.2	10.1	10.0	5.8	2.2	2.3	7.3	9.3	11.4	2.2	2.3	13.0	7.2	19.4	19.4	36.6	32.7	21.0	20.8
21	16.0	17.9	7.3	NA	4.7	NQ[f]	2.7	6.2	9.7	9.7	NQ	2.7	11.3	12.0	17.5	17.6	25.8	29.3	18.5	18.6
22	16.4	17.2	8.1	8.0	5.1	5.5	4.2	6.2	10.6	10.6	5.5	4.2	11.0	6.2	20.6	20.5	33.8	35.1	18.3	17.9

[a] All results are in mg/100 g of reconstituted powder (25 g + 200 g of water) or are as-is for RTF products.

[b] RTF = Ready-to-feed.

[c] SRM = Standard Reference Material (www.nist.gov/srm).

[d] part. hyd. = Partially hydrolyzed.

[e] NA = Not analyzed.

[f] NQ = Below the LOQ.

Table 2. Results of statistical analysis on original set of data[a]

SMPR 2012.012 requirements	n[b]	Avg., mg/100 g	≤5% RSD_r, %	≤10% RSD_R, %	HorRat
Adult nutritional RTF (high-fat)[c]	21	17.6	4.2	11.3	1.5
SRM 1849a[d]	17	8.1	3.5	3.7	0.5
Child formula powder	19	4.9	2.6	4.5	0.5
Adult nutritional powder (milk protein-based)	19	6.3	1.4	3.2	0.4
Infant formula powder (soy-based)	17	10.3	1.6	6.0	0.8
Infant formula RTF (milk-based)	19	3.5	25.6[e]	30.6[e]	3.3[e]
Adult nutritional powder (low-fat)	21	11.0	31.8[e]	33.0[e]	4.2[e]
Adult nutritional RTF (high-protein)	18	19.7	1.7	9.3	1.3
Infant elemental powder	21	34.0	7.3	11.4	1.7
Infant formula powder (partially hydrolyzed, soy-based)	22	19.5	2.4	8.0	1.1

[a] Average, RSD_r, RSD_R, and HorRat values after removal of outliers.

[b] *n* represents the number of laboratories considered in the evaluation (after removal of outliers). Twenty-two laboratories reported data.

[c] RTF = Ready-to-feed.

[d] SRM = Standard Reference Material.

[e] Results suspected to be related to sample integrity. *See* Table 4 for the final reproducibility results on these two matrixes.

the SMPR. Repeatability decreased from 31.8% in the part 2 to 1.5%, whereas reproducibility decreased from 33.0 to 6.5%; both values well below requirements. For infant formula RTF (milk-based), despite improved precision numbers (repeatability went from 25.6 to 10.7%, whereas reproducibility went from 30.6 to 13.2%), these values remained outside of the SMPR. The hypothesis that either sample spoilage (presumably of the RTF) or can mislabeling (most likely the powder) took place during the first round seemed to be confirmed.

Table 3. Full set of additional data on matrixes suspected to be spoiled (liquid) or mislabeled (powder) in the original testing[a]

Lab No.	Infant formula RTF (milk-based)[b]		Adult nutritional powder (low-fat)	
1	17.5	18.0	13.1	13.0
2	20.0	20.1	12.9	12.2
3	18.2	18.0	11.7	14.1
4	17.7	17.6	12.5	10.9
5	17.5	17.3	13.2	12.0
6	18.0	17.7	12.7	15.0
7	16.0	15.3	13.8	9.8
8	16.4	17.0	11.5	10.5
9	17.1	17.5	10.7	11.0
10	17.8	17.9	9.0	9.8

[a] All results are in mg/100 g of reconstituted powder (25 g + 200 g water) or are as-is for RTF.

[b] RTF = Ready-to-feed.

Table 4. Results of statistical analysis of the additional set of data: average, RSD_r, RSD_R, and HorRat values

SMPR 2012.012 requirements	n[a]	Avg., mg/100 g	≤5% RSD_r, %	≤10% RSD_R, %	HorRat
Infant formula RTF (milk-based)[b]	10	12.0	10.7	13.2	1.7
Adult nutritional powder (low-fat)	10	17.6	1.5	6.5	0.9

[a] *n* represents the number of laboratories in the evaluation. No outliers were removed.

[b] RTF = Ready-to-feed.

One of the participants decided to analyze the four bottles received in duplicate. To mirror this experiment, the decision was made to also analyze the samples at the Nestlé Research Center using a different method (AOAC *Official Method*℠ **985.33**). Both sets of data (data not shown) confirmed that the variability observed in the RTF sample came from sample heterogeneity not the method. These data were presented to the ERP in March 2016 and subsequently accepted.

Conclusions

The precision figures obtained during this collaborative study show that Method **2012.22** complies with the requirements set in the corresponding SMPR, and thus, that the method is fit for purpose for the analysis of vitamin C (L-ascorbic acid) in infant formula and adult/pediatric nutritionals. Both HPLC (5) or UHPLC (6) conditions are of equivalent performance, provided that suitability conditions are respected, thereby allowing for method applicability in all laboratories. These data were submitted to the ERP for review at the Mid-Year AOAC Meeting annual meeting held in March 2016 in Gaithersburg, MD. The ERP determined that the presented data met the SMPR set by SPIFAN, and thus recommended the method be granted Final Action status.

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INFANT FORMULA AND ADULT NUTRITIONALS

Quantification of Whey Protein Content in Infant Formulas by Sodium Dodecyl Sulfate-Capillary Gel Electrophoresis (SDS-CGE): Single-Laboratory Validation, First Action 2016.15

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Protein separation by sodium dodecyl sulfate-capillary gel electrophoresis, followed by UV absorption at 220 nm, allows for the quantification of major proteins in raw milk. In processed dairy samples such as skim milk powder (SMP) and infant formulas, signals from individual proteins are less resolved, but caseins still migrate as one family between two groups of whey proteins. In the first group, α-lactalbumin and β-lactoglobulin migrate as two distinct peaks. Lactosylated adducts show delayed migration times and interfere with peak separation, but both native and modified forms as well as other low-MW whey proteins still elute before the caseins. The second group contains high-MW whey proteins (including bovine serum albumin, lactoferrin, and immunoglobulins) and elutes after the caseins. Caseins and whey proteins can thus be considered two distinct nonoverlapping families whose ratio can be established based on integrated areas without the need for a calibration curve. Because mass-to-area response factors for whey proteins and caseins are different, an area correction factor was determined from experimental measurement using SMP. Method performance assessed on five infant formulas showed RSDs of 0.2–1.2% (within day) and 0.5–1.1% (multiple days), with average recoveries between 97.4 and 106.4% of added whey protein. Forty-three different infant formulas and milk powders were analyzed. Of the 41 samples with manufacturer claims, the measured whey protein content was in close agreement with declared values, falling within 5% of the declared value in 76% of samples and within 10% in 95% of samples.

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This method was approved by the AOAC Expert Review Panel for SPIFAN Nutrient Methods as First Action.
The Expert Review Panel for SPIFAN Nutrient Methods 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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Protein separation by sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE), followed by UV absorption at 220 nm, allows for the quantification of major proteins in raw milk. In processed dairy samples such as skim milk powder (SMP) and infant formulas, signals from individual proteins are less resolved, but caseins still migrate as one family between two groups of whey proteins. In the first group, α-lactalbumin (α-Lac) and β-lactoglobulin (β-Lg) migrate as two distinct peaks. Lactosylated adducts show delayed migration times and interfere with peak separation, but both native and modified forms as well as other low-MW whey proteins still elute before caseins. The second group contains high-MW whey proteins [including bovine serum albumin (BSA), lactoferrin (LF), and immunoglobulins] and elutes after the caseins. Caseins and whey proteins can thus be considered as two distinct, nonoverlapping families whose ratio can be established based on integrated areas without the need for a calibration curve. The mass-to-area response factors are different for whey proteins and caseins, and the distinct area correction factor (CF) was determined from experimental measurements using SMP samples.

This single-laboratory validation (SLV) report summarizes the results of the experiments performed to validate the *Quantification of Whey Protein Content in Infant Formulas by Sodium Dodecyl Sulfate-Capillary Gel Electrophoresis (SDS-CGE)* method following AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN)-recommended guidelines for the completion of an SLV study with reference to SPIFAN *Standard Method Performance Requirements* (SMPRs®) for whey protein-to-casein ratios.

SLV

The validation experiments, designed per SPIFAN guidelines for SLV studies (1), have demonstrated that the method is accurate, precise, specific, and linear in the analytical range, and that the method is suitable for its intended purpose. A summary of all validation experiments and results can be found in Table **2016.15A**. The samples used during the execution of the validation testing are detailed in Table **2016.15B**.

Table 2016.15A. Summary of validation characteristics, acceptance criteria, and results

Parameter	Acceptance criteria (SMPR)	Results
Applicability	Determination of total whey proteins, including hydrolyzed forms, as the percentage of protein content (protein content as defined by the appropriate regulatory agencies). To be applicable to milk-based infant formula products (including those from bovine milk and, if possible, milk of other species and products containing hydrolyzed casein).	Applicable for the determination of whey percentage as the total protein in bovine milk-based infant formula. This method is not applicable to the analysis of hydrolyzed protein-based infant formulas.
Accuracy	Percentage recovery must be within the theoretical range of 95–105%.	Recovery range was 97.4–106.4%.
Repeatability precision	RSD ≤ 3.0% for whey protein g/100 g protein	RSD was 0.3–1.2% in five different infant formula sample types.
Intermediate precision	RSD ≤ 3.0% for whey protein g/100 g protein	RSD was 0.5–1.1% in five different infant formula sample types.
Specificity: Matrix interference	E-grams from injections of purified water and processed formulation matrix without protein ingredients must be evaluated for the presence of peaks at the migration times corresponding to analyte protein-related peaks.	No interfering peaks were observed for purified water or the processed formulation matrix.
LOQ	≤10 whey protein g/100 g protein	20% of total protein in infant formulas
Linearity	R^2 must be ≥0.99. The residuals on the residual plot should be randomly distributed around zero.	Linearity of R^2 of 0.993–0.999 for the area ratio of whey protein to casein
		Logarithm of R^2 of 0.993–0.996 for whey protein as the percentage of total protein
		Residuals on the residual plot were randomly distributed around zero.
Range		Range of 20–100% for whey protein in total protein in infant formulas in the tested linear range

Table 2016.15B. Validation test sample description

Sample	Description
Infant formula 1	First-age infant formula with a manufacturer claim of 60% whey protein, manufactured with sweet whey ingredient
Infant formula 2	First-age infant formula with a manufacturer claim of 60% whey protein, manufactured with sweet whey ingredient
Infant formula 3	First-age infant formula with a manufacturer claim of 65% whey protein, manufactured with α-Lac-enriched whey
Infant formula 4	First-age infant formula with a manufacturer claim of 70% whey protein, manufactured with CGMP-reduced whey
Infant formula 5	Third-age infant formula with a manufacturer claim of 40% whey protein, manufactured with sweet whey ingredient
SMP	20% whey protein
Sweet whey	Demineralized whey, 13% total protein

AOAC Official Method 2016.15
Quantification of Whey Protein Content in Infant Formulas by Sodium Dodecyl Sulfate-Capillary Gel Electrophoresis (SDS-CGE)
First Action 2016

[Applicable for the determination of the whey-to-casein protein ratio, ranging from 20:80 to 80:20, in bovine milk-based infant formula powders. This method is not applicable to the analysis of hydrolyzed protein-based infant formulas.]

Caution: Correct personal and environmental safety standards must be used while performing this analytical method. Laboratory personnel handling solvents, acids, and reagents should be knowledgeable of their potential hazards. Consult the Material Safety Data Sheets for information on hazards and how to take proper precautions. Only transfer solvents and acids inside efficient fume hoods and extractors. Ensure all glassware is free from chipping and hairline cracks.

A summary of all validation experiments and results can be found in Table **2016.15A**. The samples used during the execution of the validation testing are detailed in Table **2016.15B**.

A. Principle

In sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE), proteins in infant formula samples are denatured by anionic surfactant SDS and reduced by β-mercaptoethanol. The SDS-bonded electrically charged proteins migrate in an electrical field filled with a separation gel and are detected by UV at 220 nm^2. Caseins and whey proteins are separated as two distinct nonoverlapping groups of peaks whose ratio can be established based on integrated areas without the need for a calibration curve. A mass-to-area correction factor (CF) of 1.4 was used for whey proteins versus caseins in the calculation of whey protein content.

B. Apparatus

(a) *ProteomeLab PA 800 Plus*.—Beckman Coulter, Inc. (Fullerton, CA) or equivalent, equipped with a UV detector set at 220 nm. Peak area integration can be achieved by using any suitable software (e.g., Waters Empower, Beckman 32 Karat, or equivalent).

(b) *Bare fused-silica capillaries*.—50 µm id × 20 cm (e.g., Model 338451; Beckman Coulter, Inc.).

C. Reagents

(a) *SDS-MW gel buffer*.—Part No. A30341 (Beckman Coulter, Inc.); recipe readily supplied by the vendor.

(b) *SDS-MW analysis kit (2)*.—Part No. 390953 (Beckman Coulter, Inc.), including bare fused-silica capillaries (50 µm id × 20 cm), SDS-MW sample buffer (100 mM Tris–HCl, pH 9.0; with 1% SDS), 10 kDa protein internal standard (IS), acidic

wash solution (high-purity, 0.1 N HCl), basic wash solution (high-purity 0.1 N NaOH), and an SDS-MW size standard (10–225 kDa, 16 mg/mL).

(c) *Protein IS*.—10 kDa, Part No. A26487 (Beckman Coulter, Inc.).

(d) *Water*.—LC grade.

(e) *β-Mercaptoethanol*.—Part No. M7154 or M6250 (Sigma).

D. Preparation of System Buffer Trays and Standard and Sample Solutions

(a) To prepare the system buffer trays, follow the steps in Figure **2016.15A** and load reagents into the system inlet (lower left panel) and outlet (lower right panel). Use 6 × 6 buffer trays, following the configuration illustrated in the panels.

(b) Either weigh 135 ± 5 mg skim milk powder (SMP; protein content around 37%) or 500 ± 20 mg infant formula powder (protein content around 11%) into a 15 mL centrifuge tube.

(c) Dissolve the sample and dilute to a 5 mL volume with deionized (DI) water. Mix each tube on a vortex mixer until the samples are homogeneously dissolved. Each final solution should contain about 10–15 mg/mL protein.

(d) Prepare the sample running presolution by mixing 1% SDS sample solution with 10 kDa IS peptide using an 84:1 ratio based on the total number of samples to be analyzed in the sample set (90 µL/sample).

(e) Pipet 10 µL of each sample solution into separate 2.0 mL microcentrifuge vials.

(f) Sequentially add 85 µL sample running presolution and 5 µL β-mercaptoethanol to each microcentrifuge vial. Mix well before heating the vials in a water bath at 100 ± 5°C for 10 min. Cool down to room temperature, then centrifuge for 1 min at about 7000 rpm.

(g) Mix on a vortex mixer before transferring each sample into their corresponding injection vials.

E. Sample Analysis

(a) Set up an optimized separation method for the batch analysis of up to 24 samples at a time, including a buffer blank (10 µL DI water), an MW size standard, and an SMP sample.

(b) For each separation cycle (40 min), precondition the capillary first with basic wash solution, followed by acidic wash solution, DI water, and SDS gel buffer.

(c) Introduce the samples electrokinetically by applying voltage at –5 kV for 20 s.

(d) Perform electrophoresis at constant voltage with an applied field strength of –497 V/cm and the capillary thermostatted to 25°C using recirculating liquid coolant.

(e) The current generated should be approximately 27 µA.

(f) Program the system to automatically replenish all reagents through incremental increases in buffer array after every eight cycles.

(g) Test system suitability using the MW marker. Acceptance criteria for the system suitability are as follows: The migration time of the IS should be 12.3 ± 0.5 min, and the migration pattern and migration times of the seven MW markers (10, 20, 35, 50, 100, 150, and 225 kDa) should completely separate within 30 min using this method. *See* Figure **2016.15B**.

(h) Acceptance criteria for the separation cycle are as follows: The migration time of the IS should be 12.3 ± 0.5 min, the degree of baseline drop from the migration time of the IS to the peak valley between the end of casein and the peak of immunoglobulin heavy chain (Ig H) and bovine serum albumin (BSA) should be no more than 25% of the height of the IS of the sample.

(i) To integrate SMP and infant formula electrophoregrams (e-grams), set the baseline at 0.4 min before the IS peak to the valley between the end of the κ-casein peak and the Ig H

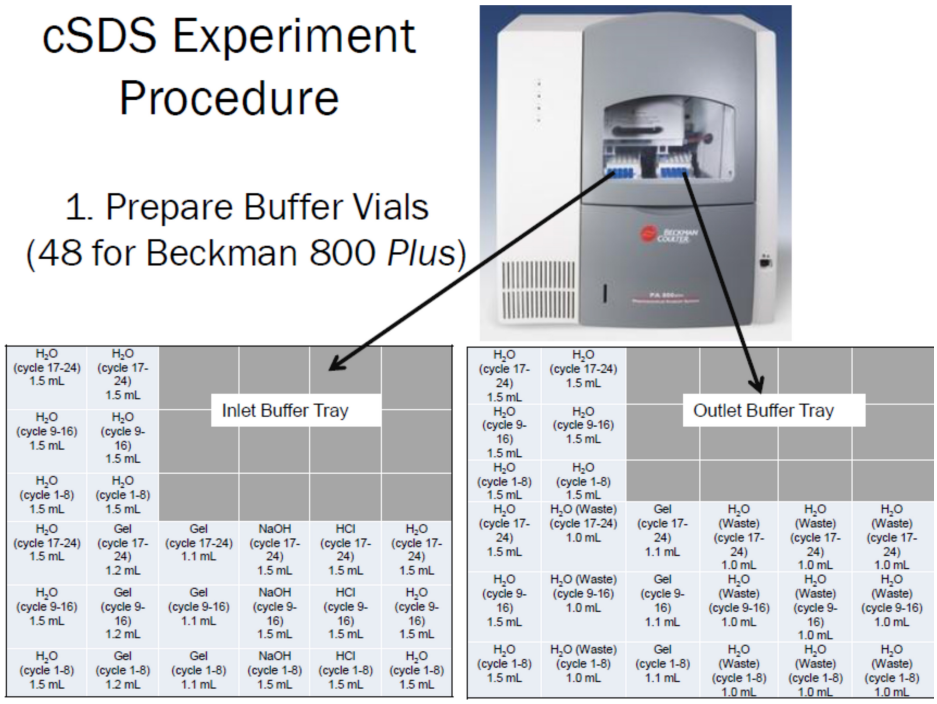

Figure 2016.15A. Preparation of system buffer trays.

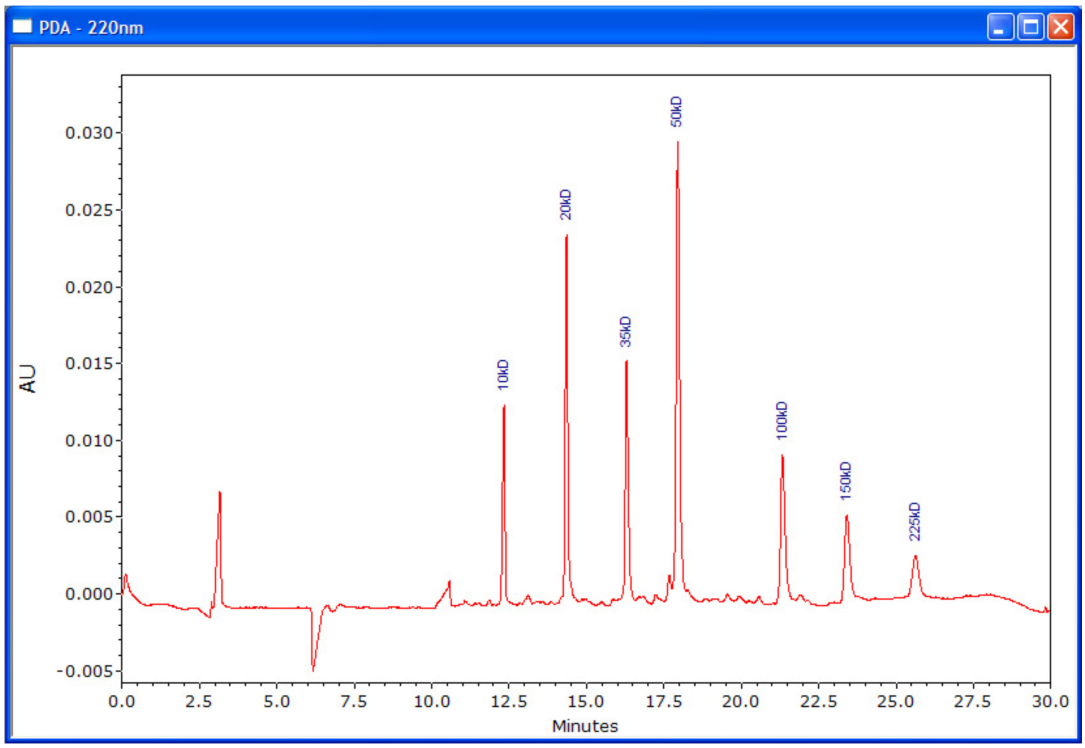

Figure 2016.15B. Separation of the protein MW size standard.

and BSA peak; perform a manual integration from the valley between the end of the κ-casein peak and the peak of Ig H and BSA to the end of the last peak in the e-grams (at least 9 min after the peak of the 10 kDa IS).

(j) To determine the casein region, set the start time for casein integration just before the β-casein peak in the e-gram of the SMP (about 3.1 min after the peak of the 10 kDa IS). Referencing the SMP, identify the β-casein peak in the infant formula samples, then set the start time of the casein region in the infant formula to just before the β-casein peak. Set the end time at the valley between the end of the κ-casein peak and the Ig H and BSA peak (about 7.0 min after the 10 kDa IS).

F. Calculations

(a) To calculate whey protein content, separately sum the peaks in the following three regions: two at each end of the e-gram (smaller and larger whey proteins) and one in the middle. The middle region corresponds to casein proteins (A_{cn}), and the two others are summed together to obtain the whey proteins (A_w).

(b) Whey protein content is calculated using the following equations:

$$\text{Percentage of whey protein} = \frac{A_{w,c}}{A_{w,c} + A_{cn}} \quad (1)$$

$$A_{w,c} = A_w \times 1.4 \quad (2)$$

where A_w = total integrated areas of whey components; $A_{w,c}$ = corrected integrated area of whey components; A_{cn} = integrated area of casein components; and 1.4 = CF to account for the difference between the mass-to-area ratio of whey and casein proteins.

Results and Discussion

Specificity

(a) *Reagent blank*.—Each sample sequence was started with purified water as a blank. The blank e-gram is shown in Figure 1 (gray line). No peaks were detected after the 10 kDa peptide internal standard (IS). There was no significant interference from other components in the protein region.

(b) *Placebo test*.—To test for the presence of interference from nonprotein components in infant formulas, a placebo infant formula trial sample that contained all of the ingredients that are typical first-age formulas, except protein (vitamins, minerals, fat, and carbohydrates), was manufactured. SDS-CGE did not detect significant peaks at any of the protein regions in the e-gram (Figure 1, black line).

(c) *Specific protein migration time and migration pattern of whey proteins and caseins*.—The SDS-CGE method can separate individual whey and casein protein standards very well, as demonstrated with standard solutions containing five major whey proteins (Figures 2 and 3) or four casein proteins (Figure 4), as well as with fresh raw milk (Figure 5). Protein phosphorylation and glycosylation delay casein migration times relative to their molecular sizes (Figure 6). Protein glycation—the nonenzymatic sugar modification of amines and the early stage of a Maillard reaction—occurs during the mixing and heating of milk proteins with lactose (3), which results in the splitting of several individual milk proteins into several peaks representing the modified protein glycoforms. This was seen for α-Lac and β-Lg, where splitting was observed in a commercial sweet whey protein ingredient (Figure 7) and by comparing the casein peaks in fresh milk and in an SMP sample (Figures 5 and 8). Although glycation prevents the complete separation of all proteins individually, whey proteins

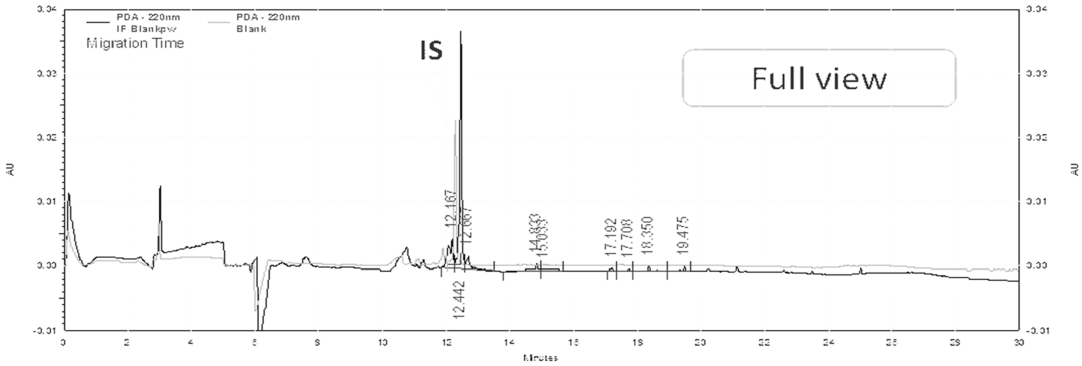

Figure 1. E-grams of pure water blank (gray line) and placebo (processed; black line). Compared with the reagent blank (gray line), there was no significant interference from nonprotein components in the protein range.

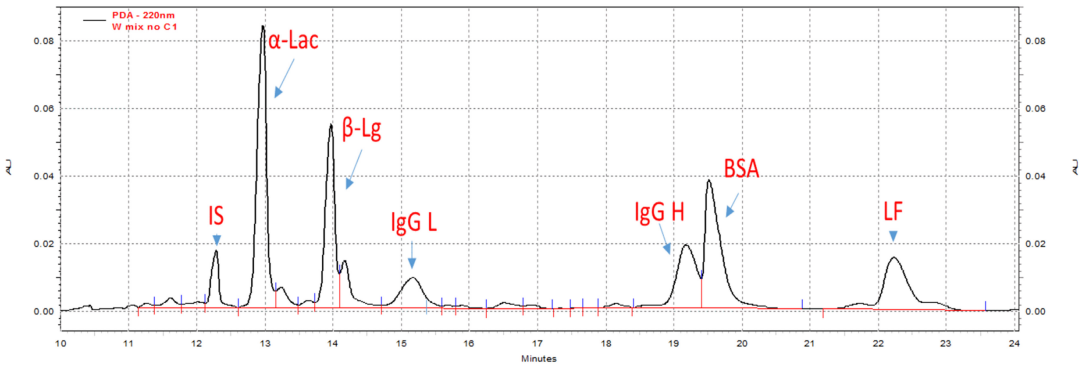

Figure 2. E-gram of the five major whey protein standards mixed (group 1): α-Lac, β-Lg, bovine immunoglobulin G [IgG light (L) and heavy (H) chains], BSA, and LF. All standards were from Sigma.

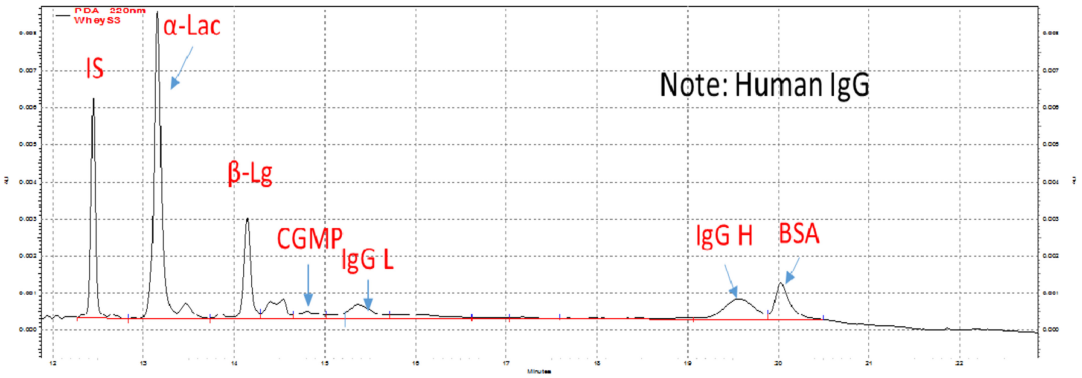

Figure 3. E-gram of the five major whey protein components standards mixed (group 2): α-Lac, β-Lg, CGMP, immunoglobulin G [IgG light (L) and heavy (H) chains], and BSA. All standards were from Sigma, except for CGMP, which was from Arla Ingredients, Inc.

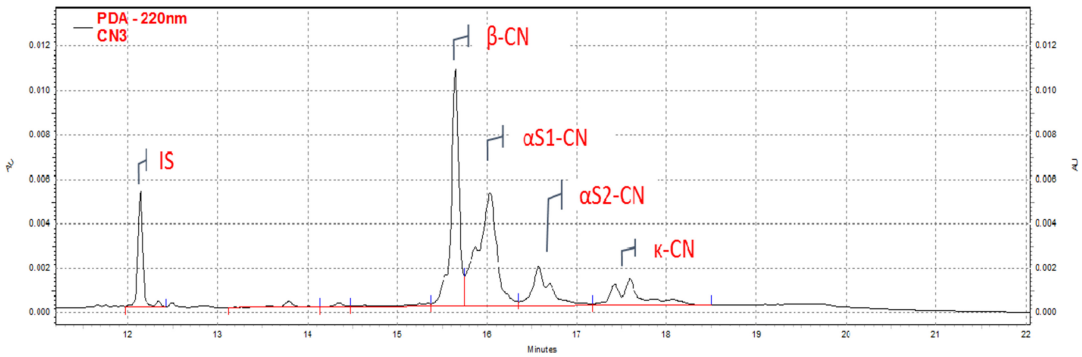

Figure 4. E-gram of the casein protein standard from Sigma.

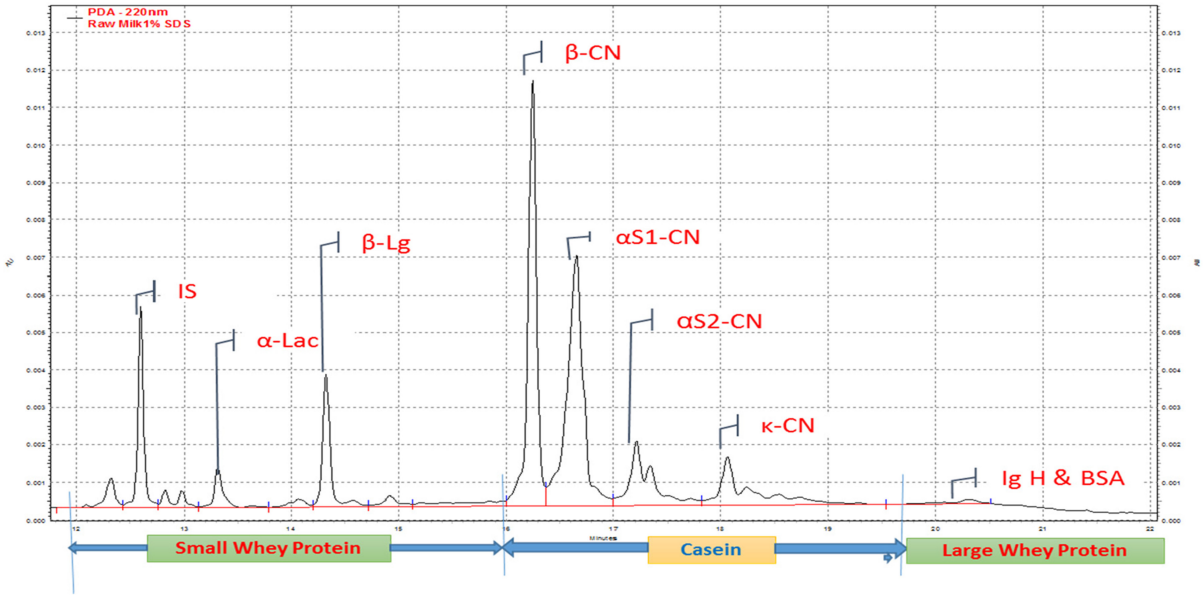

Figure 5. E-gram of raw milk.

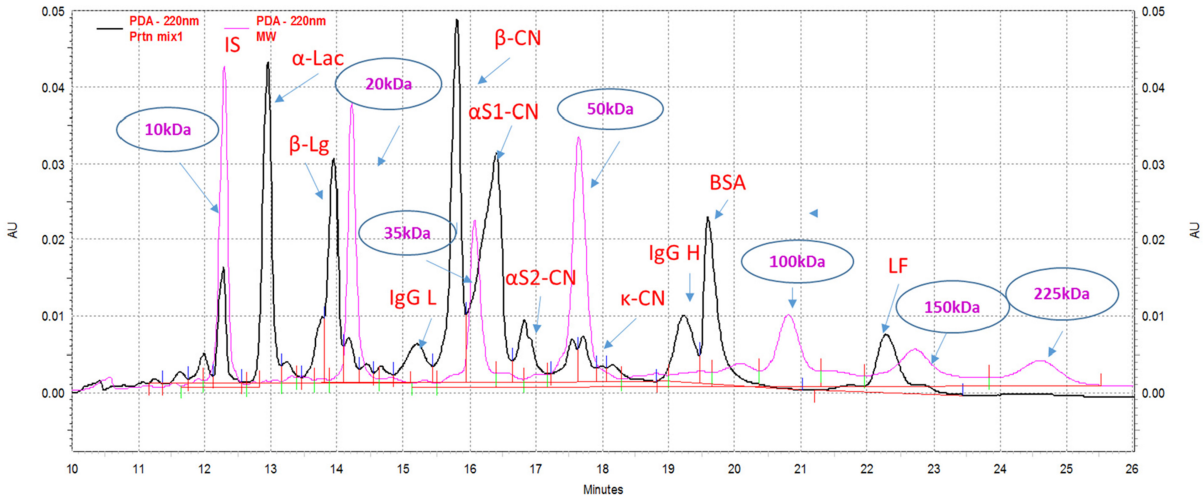

Figure 6. E-gram of major whey proteins and casein proteins (black) compared with the MW marker (circled kDa values).

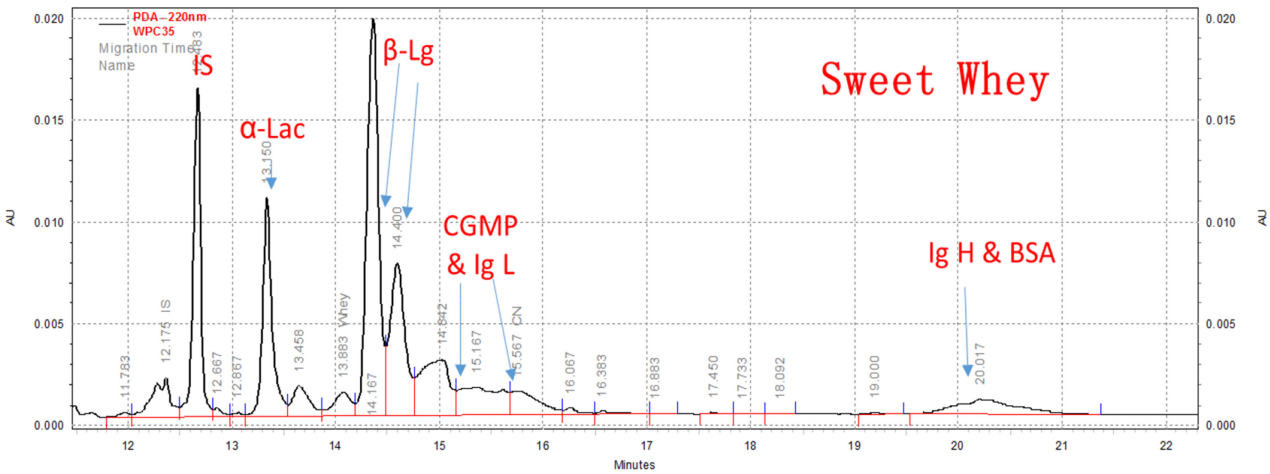

Figure 7. Typical e-gram of sweet whey ingredients.

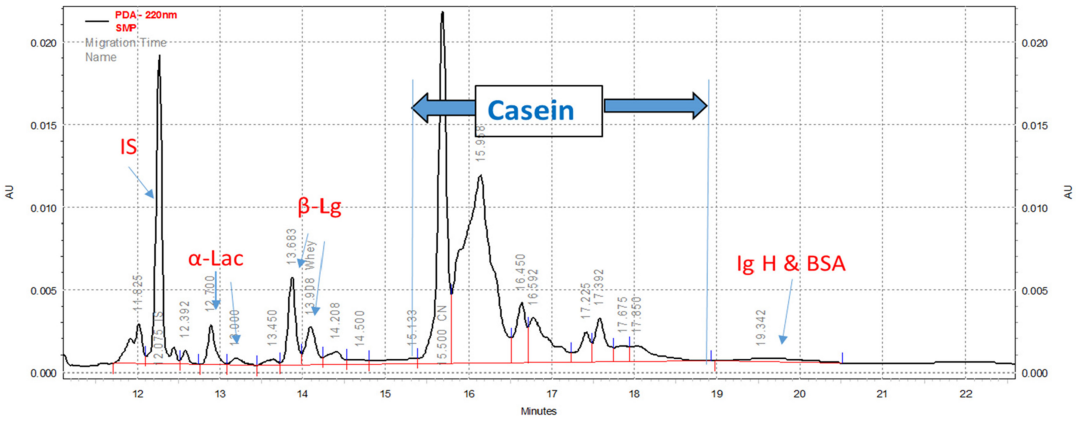

Figure 8. Typical e-gram of SMP.

still migrate as two groups either before or after the caseins. Caseins were eluted as a single group between light and heavy chains of immunoglobulins, where almost no major whey proteins were found (Figure 8).

Linearity

Linearity data were obtained by spiking seven different levels of whey protein ingredients into a fixed level of SMP. Whey protein content was designed to range from 20% (no added whey) to about 80% in the mixtures of whey and skim milk. The typical spiking scheme is presented in Table 1.

Analyses were performed in duplicate on a single day, as well as in single analyses on 3 separate days. The relationship between the amount of added whey protein and the whey protein-to-casein ratio proved to be linear, and the coefficients of determination (R^2) were all higher than 0.9900 (Table 2). The relationship between the amount of added whey protein and the percentage of added whey in the total protein proved to be logarithmic; the R^2 were also all higher than 0.9900 (Table 2). Typical linearity relationships are shown in Figure 9.

Table 1. Testing scheme for linearity and accuracy (whey protein ranging from 20 to 80%)

			Protein		
Ingredients[a]	Weight, mg	DI water, mL[b]	%	mg/mL	Lot No.
SMP	84.6	2.00	38.5	16.3	DY19
WPC35	162	1.00	35.2	57.0	C22JUL15J1
Experiment[c]	SMP, µL	WPC35, µL	DI water, µL	Total, µL	Whey content, %
WPCL0	60	0	60.0	120.0	21.2
WPC35L1	60	5	55.0	120.0	37.6
WPC35L2	60	10	50.0	120.0	48.0
WPC35L3	60	20	40.0	120.0	60.4
WPC35L4	60	30	30.0	120.0	67.5
WPC35L5	60	40	20.0	120.0	72.2
WPC35L6	60	60	0.0	120.0	77.8

[a] WPC = Whey protein concentrate.

[b] DI = Deionized.

[c] L0–6 = Levels 0–6.

Table 2. Summary of R^2 and the equations for seven levels of whey protein in total proteins for the whey-to-casein ratio and added whey protein as the percentage of total protein

	Day 1: Avg. for two replicates	Day 2: Single	Day 3: Single	Day 4: Single
	Whey/casein			
R^2	0.9931	0.9990	0.9980	0.9984
Equation	$y = 0.0378x + 0.2573$	$y = 0.0421x + 0.2119$	$y = 0.0429x + 0.2013$	$y = 0.044x + 0.2232$
	Percentage whey added			
R^2	0.9961	0.9942	0.9957	0.9928
Equation	$y = 16.909 \ln(x) - 12.42$	$y = 16.564 \ln(x) - 9.7994$	$y = 17.576 \ln(x) - 13.359$	$y = 16.339 \ln(x) - 8.6141$

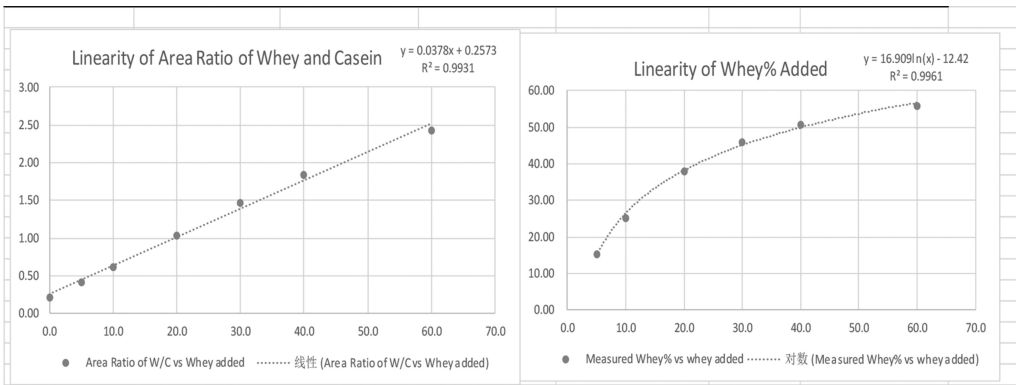

Figure 9. Typical linearity relationships between the area ratio of whey to casein and measured whey percentage versus whey protein amount added.

Table 3. Spike-recovery data obtained in duplicate for different levels on 1 day

	Integrated area		Measured		Theoretical	
Spike level	Total whey	Casein	Whey, %	Whey, % spiked	Whey, % spiked	Recovery, %
0	90447	431555	21.3			
	95042	450546	21.4			
1	467929	454430	59.0	37.7	39.0	96.6
	489937	464188	59.6	38.3	39.0	98.1
2	686816	457990	67.7	46.4	46.2	100.5
	699775	481562	67.0	45.7	46.2	99.0
3	823437	448464	72.0	50.7	50.8	99.7
	847557	455946	72.2	50.9	50.8	100.2
4	1181386	467897	77.9	56.6	56.5	100.2
	1179619	504671	76.6	55.3	56.5	97.8

Accuracy (Spike Recovery)

Accuracy was evaluated in samples where four levels of sweet whey ingredient were spiked into the same level of SMP by comparing the theoretically calculated percentage whey values with the measured values obtained using equations 1 and 2 (for skim milk, where no whey proteins were added, the CF was set to 1.29). The results are listed in Tables 3 and 4. The SMPs and whey protein ingredients used in Tables 3 and 4 are from different lot numbers.

Precision

Two independent sample preparations were tested on each day for 6 separate days for the following: three infant formula samples (shown in Table 5), one SMP sample, and one sweet whey (demineralized) sample (shown in Table 6). Two other infant formula samples were tested by preparing and analyzing six replicates each day on 3 different days using this method (shown in Table 7). The percentage of whey protein was reported to one decimal place for individual and averaged replicates. The SD and percentage RSDs were calculated and also reported to one decimal place. Data are presented in Tables 5–7. The typical e-grams for each infant formula sample are presented in Figures 10–14.

The molar attenuation (or molar extinction) coefficient, reflected as the mass-to-area ratio at 220 nm, is an intrinsic

Table 4. Spike-recovery data obtained in singlet for different levels on 3 different days

		Area		Measured		Theoretical	
Level	Day	Total whey	Casein	Whey, %	Whey, % spiked	Whey, % spiked	Recovery, %
0	1	112590	581315	20.0			
	2	134644	668598	20.6			
	3	125910	626821	20.6			
1	1	544500	636291	54.5	34.5	32.1	107.6
	2	528392	647887	53.3	32.7	31.3	104.4
	3	544475	625202	54.9	34.4	33.0	104.2
2	1	632182	609700	59.2	39.2	37.3	105.0
	2	654331	605001	60.2	39.6	36.6	108.1
	3	698583	633572	60.7	40.1	38.3	104.8
3	1	805608	630879	64.1	44.1	41.4	106.6
	2	789708	615699	64.2	43.6	40.8	106.9
	3	836902	621176	65.4	44.8	42.3	105.8
4	1	939097	642338	67.2	47.2	44.7	105.6
	2	930074	631568	67.3	46.7	44.1	105.8
	3	906509	594583	68.1	47.5	45.6	104.3

Table 5. Repeatability and intermediate precision for three infant formulas

	Formula/Replicate					
Day	1/1	1/2	2/1	2/2	3/1	3/2
1	61.0	60.8	55.9	57.2	64.4	64.2
2	59.7	59.8	56.5	56.1	64.4	64.7
3	60.5	59.3	56.2	56.1	64.4	65.1
4	60.0	59.9	56.1	56.6	64.2	64.2
5	59.5	58.8	56.4	56.5	64.8	65.2
6	59.3	59.7	57.1	57.8	64.9	64.8
Avg.	59.9		56.4		64.7	
RSD_r, %	0.5		0.8		0.3	
RSD_R, %	1.0		0.8		0.7	

Table 6. Repeatability and intermediate precision for SMP and demineralized sweet whey ingredient

Day	SMP[a]	SMP[a]	DW[b]	DW[b]
1	21.7	21.2	95.5	98.6
2	20.1	19.8	98.3	97.9
3	21.8	21.4	97.4	97.6
4	21.6	21.9	97.0	98.3
5	22.2	22.4	98.5	99.2
6	22.1	22.4	98.8	97.9
Avg.	21.7		97.9	
RSD_r, %	1.5		0.9	
RSD_R, %	3.8		1.0	

[a] Because SMP is not processed like infant formula, a CF of 1.29 was used.

[b] Because whey protein contains almost no caseins, no CF was used.

Table 7. Repeatability and intermediate precision for two infant formulas

	Rep. 1	Rep. 2	Rep. 3	Rep. 4	Rep. 5	Rep. 6	Avg.	SD	RSD
Day	Formula 4								
1	71.2	70.5	70.7	71.1	71.1	70.8	70.9	0.3	0.4
2	71.0	70.7	71.2	70.7	70.7	70.8	70.9	0.2	0.3
3	70.6	70.4	70.1	70.3	70.5	70.3	70.3	0.2	0.2
Total							70.7	0.3	0.5
	Formula 5								
1	40.7	41.8	41.6	41.3	41.1	41.7	41.4	0.4	1.0
2	41.7	42.4	42.5	41.2	42.2	42.2	42.0	0.5	1.2
3	41.8	42.0	42.0	42.1	41.9	41.6	41.9	0.2	0.4
Total							41.8	0.5	1.1

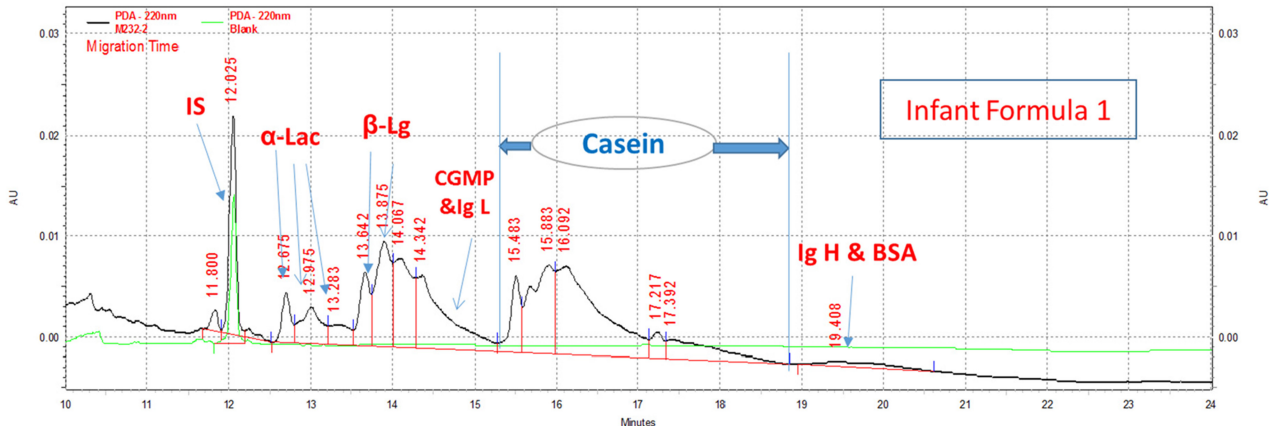

Figure 10. E-gram of infant formula 1 with sweet whey ingredient (60% whey claim).

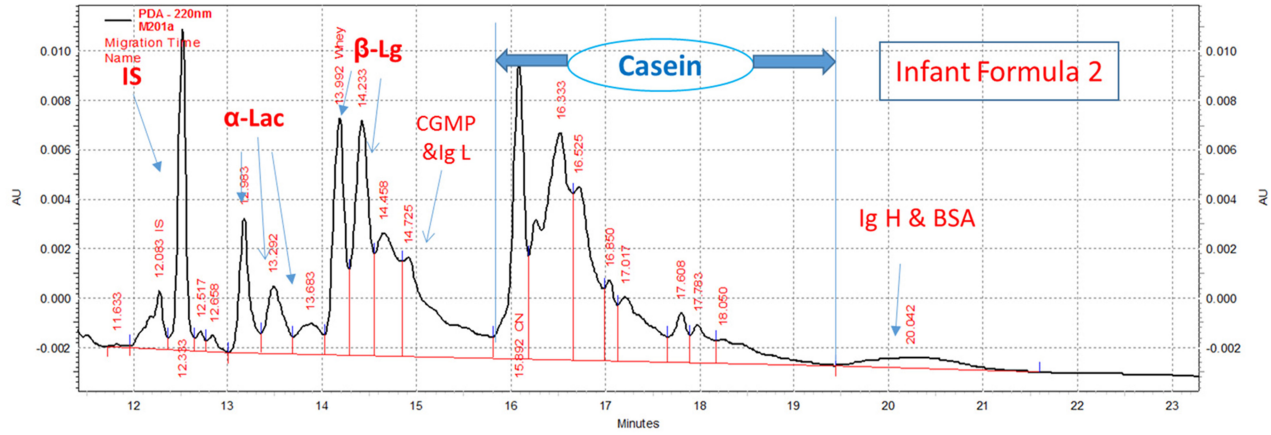

Figure 11. E-gram of infant formula 2 with sweet whey ingredient (60% whey claim).

property of proteins and depends on the proteins' amino acid sequence and molecular structure status. Unfortunately, no literature is currently available regarding whey protein and casein ratios under SDS-CGE conditions, nor for proteins after infant formula processing. In contrast, the mass ratio of whey proteins to caseins is well established (4, 5; Table 8) and can be calculated as 26.9% (whey proteins versus caseins) in bovine milk and SMP.

To correct for the difference between the mass-to-area ratios of whey proteins and caseins, 13 SMP samples from different batches and suppliers were analyzed by SDS-CGE with 50 measurements. The two whey protein areas and the one casein area were integrated, and the area percentage ratio of whey proteins to caseins was established at 20.8% (Table 9).

The mass-to-area CF for whey proteins relative to caseins was obtained by comparing the whey-to-casein mass percentage

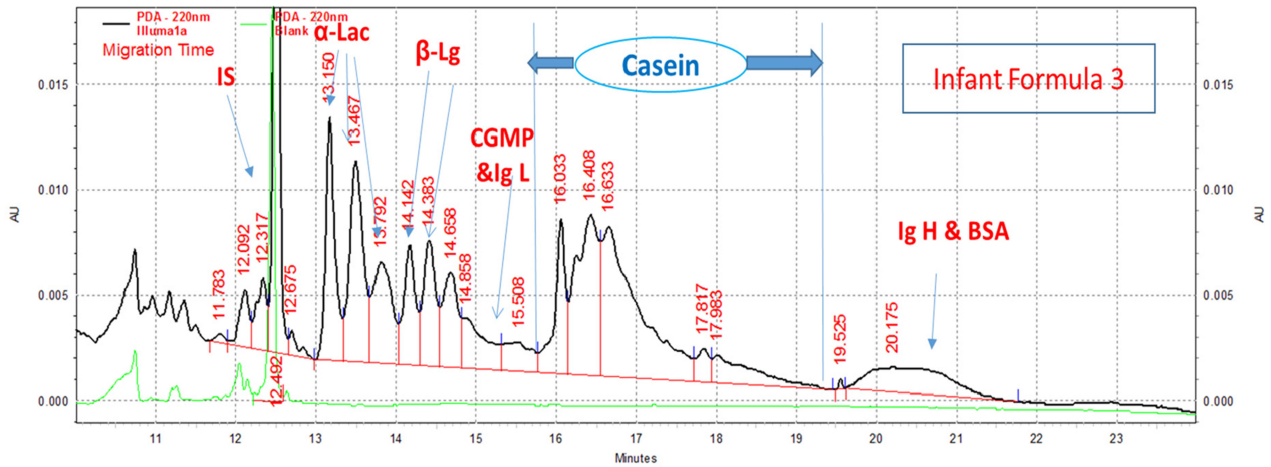

Figure 12. E-gram of infant formula 3 with α-Lac-enriched whey ingredient (65% whey claim).

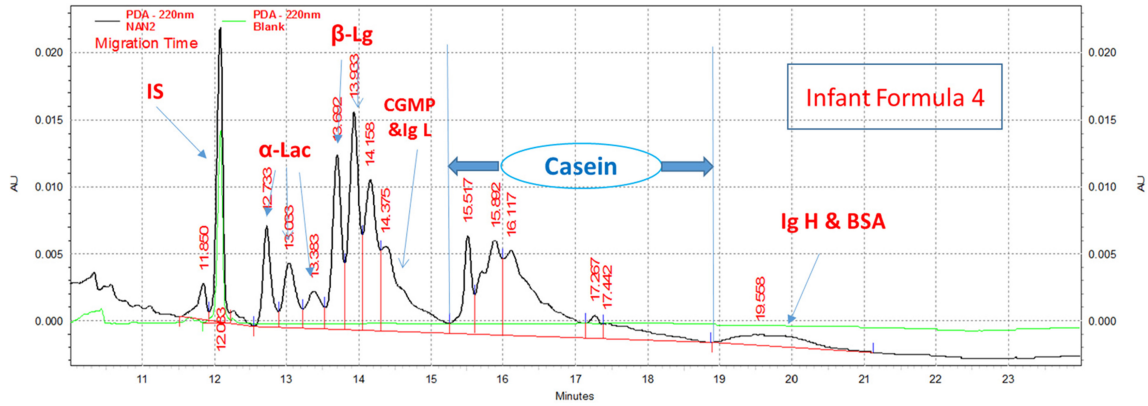

Figure 13. E-gram of infant formula 4 with CGMP-reduced sweet whey ingredient (70% whey claim).

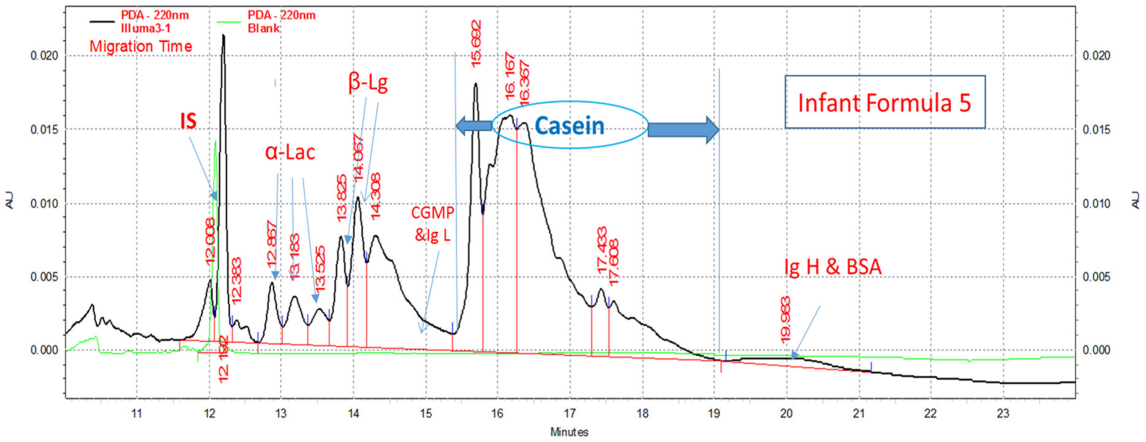

Figure 14. E-gram of infant formula 5 with sweet whey ingredient (40% whey claim).

Table 8. Protein profile of bovine milk and calculated whey protein as the mass percentage of casein (4, 5)

		Whole milk content	
Protein	MW, kDa	g/L (5)	%
αS1-casein	23.69	10.0	30.3
αS2-casein	25.31	2.6	7.9
β-Casein	23.97	9.3	28.2
κ-Casein	21.30	3.3	10.0
γ-Casein	20.59	0.8	2.4
α-Lac	14.19	1.2	3.6
BSA	67.41	0.4	1.2
Immunoglobulin	160.00	0.8	2.4
Peptone 5	12.43	0.5	1.5
Peptone 3	17.89	0.3	0.9
LF	78.06	0.1	0.3
Milk fat globule membrane		0.4	1.2
β-Lg	18.27	3.3	10.0
CGMP	9.15		
Sum		33.0	100.0
Casein		26.0	78.8
Whey		7.0	21.2
Whey as percentage of casein		26.9	26.9

ratio from the literature with the area percentage ratio obtained with the SDS-CGE method. Based on the literature mass percentage ratio (26.9%) and the experimental area percentage ratio (20.8%), a CF of 1.29 should be applied to the integrated signal of whey proteins.

To evaluate the impact of the infant formula manufacturing process on the area CF of whey proteins to caseins, a whey protein-dominant infant formula was manufactured. Two samples were taken; one before processing and one after. The test results are listed in Table 10 and indicate that processing further increased this ratio 1.11-fold. Therefore, a final CF of 1.4 for whey protein-to-casein area for infant formulas was chosen (Table 9).

Forty-three infant formulas manufactured by both Chinese and international manufacturers with different whey ingredients, including regular sweet whey, α-Lac-enriched whey, LF-added whey, and casein glycomacropeptide (CGMP)-reduced whey, were analyzed and compared with manufacturers' claims (Table 11). The results show that among the 41 samples with manufacturers' claims, measured whey content was in close agreement with declared value: within 5% of the declared value for 31 (76%) samples and within 10% for 37 (90%) samples. Two infant formulas did not contain added whey protein; hence, a factor of 1.29, not 1.4, should be used. Taking this into account, 39 (95%) samples were within 10% of the declared value.

Table 9. Measured results of whey protein as the area percentage of caseins for different batches of SMP samples from different suppliers by SDS-CGE and the calculated area CF of whey proteins to caseins

Whey as percentage of casein (mass)			
Literature (4, 5)		26.9	
Whey as percentage of casein (area)			
Lot No.	*n*[a]	Avg.	SD
EY06	3	20.02	0.58
CY25	2	20.62	0.35
DY06	2	20.25	0.17
DY19	4	20.58	0.30
DY21	5	20.44	0.81
DY29[b]	24	21.32	1.00
M023	2	18.84	0.09
M075	2	22.26	1.02
M208	2	21.40	1.16
MSK	1	22.27	
SMP DN1	1	21.53	
SMP DN2	1	21.34	
SMP DN3	1	19.79	
Avg.[c]		20.81	
SD[d]		0.99	
CV, %		4.78	
CF		1.29	
Process impact[e]		1.11	
Final CF		1.4	

[a] *n* = Number of measurement.

[b] Four different batches of capillaries with two different sets of reagent kits on 12 different days.

[c] Avg. = Average.

[d] SD = Standard deviation.

[e] Obtained by evaluating processed and finished infant formula product (Table 10).

Table 10. Comparison of the area percentage of whey protein between the times after compounding and after spray-drying during the processing of formula trial samples

	Area			
	Whey	Casein	Whey/casein	CF
Before processing	306543	257994	1.19	1.11
After processing	337097	314112	1.07	

Conclusions and Recommendations

The SDS-CGE method is capable of accurately determining the ratio of whey to casein in infant formulas manufactured using different whey ingredients. Because whey and casein proteins, as groups, have distinct migration times, the measurements will not miss individual proteins. As a consequence, absolute quantification of individual or total proteins is not necessary.

It was recommended that the method be further validated by conducting a multilaboratory study. This would generate valuable method performance data, including RSD_R, further enhancing the potential of this method for use in a routine QC environment.

Table 11. Measured whey protein content in 43 different infant formulas made by both local and international manufacturers

Product	Manufacturer whey claim, %[a]	Measured whey, %			% of claim
		n	Avg.	SD	
1	60	12	59.9	0.50	100
2[b]	70	18	70.7	0.34	101
3	60–65 (62.5)	12	56.4	0.80	90
4	40	18	41.8	0.47	104
5[c]	65	12	64.7	0.30	99
6	60	4	59.6	0.23	99
7	60	4	59.7	0.66	99
8	65	4	65.9	0.41	101
9	65	3	63.2	0.34	97
10[b]	70	3	71.2	0.84	102
11[d]	60	3	62.9	1.15	105
12	60	2	57.3	0.09	95
13	60	2	64.4	0.10	107
14	60	2	63.2	0.27	105
15	60	2	63.7	1.26	106
16	N/L[e]	2	20.2	0.92	
17	70	2	69.0	1.03	99
18[c]	65	2	62.2	1.69	96
19	65	2	65.5	1.03	101
20	70	2	70.6	1.51	101
21	61	2	61.5	1.43	101
22	70	1	62.4		89
23	>60 (65)	1	67.5		Pass[f]
24	70	1	70.2		100
25	70	1	65.0		93
26	60	1	67.3		112
27	60	1	62.9		105
28	60	1	59.3		99
29	62	1	64.9		105
30	60	1	62.4		104
31	61	1	64.4		106
32	60	1	64.0		107
33	60	1	60.5		100
34	70	1	70.0		100
35	N/L	1	42.1		
36	60	1	57.4		96
37	60	1	58.9		98
38	64	1	66.4		104
39	60	1	62.7		105
40	60	1	60.0		100
41	60	1	60.7		101
L1[g]	21	2	22.6	2.43	108
L2[g]	21	2	22.5	1.71	107

[a] Numbers in parentheses represent value considered.

[b] CGMP-reduced sweet whey formula.

[c] α-Lac-enriched formula.

[d] LF-enriched formula.

[e] N/L = Not labeled.

[f] Conform to claim.

[g] The claim of 21.2% comes from the value for SMP, not a real claim.

Acknowledgments

We thank Hongxu Chen and Jianhua Wu (AB Sciex) for providing the PA 800 Plus instrument and instrument maintenance, Chao Wu (Hilmar Ingredients) for helping us understand the meaning of split peaks for infant formula proteins, Longfei Wang (Nestlé Food Safety Institute, Beijing) for providing the laboratory facilities, and Wyeth Nutrition Suzhou for supplying pilot-scale placebo infant formula samples and samples with different protein ingredients.

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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.

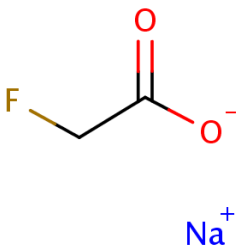

Figure 2015.02A. Sodium fluoroacetate.

(**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.

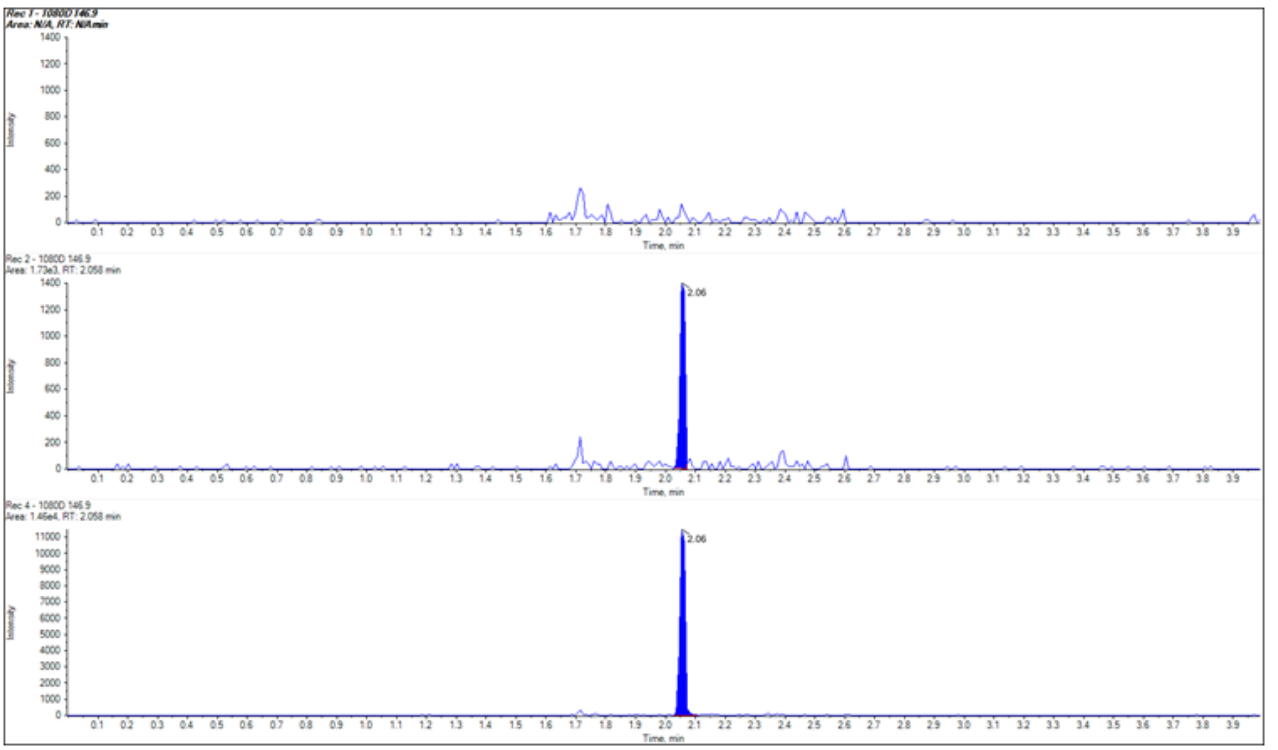

Figure 2015.02B. Chromatograms of confirmation ion for recovery 1 (matrix blank; top), recovery 2 (0.1 µg/kg, ≈LOQ; middle), and recovery 4 (1.0 µg/kg, LOR; bottom).

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)

OFFICIAL METHODS

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

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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.

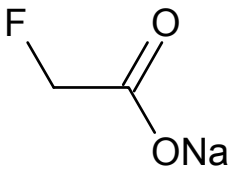


Figure 2015.03A. Chemical structure of sodium fluoroacetate ($NaFC_2H_2O_2$; CAS No. 62-74-8; MW 100 g/mol).

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

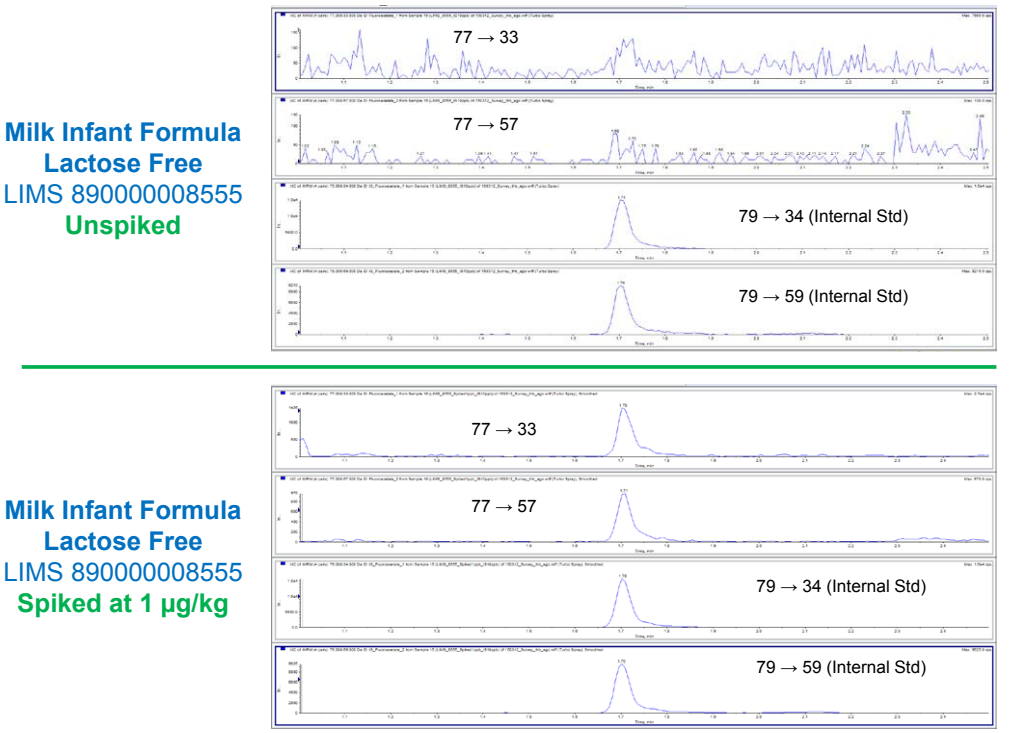


Figure 2015.03B. LC-MS/MS chromatograms of a powdered milk-based infant formula (lactose free) unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

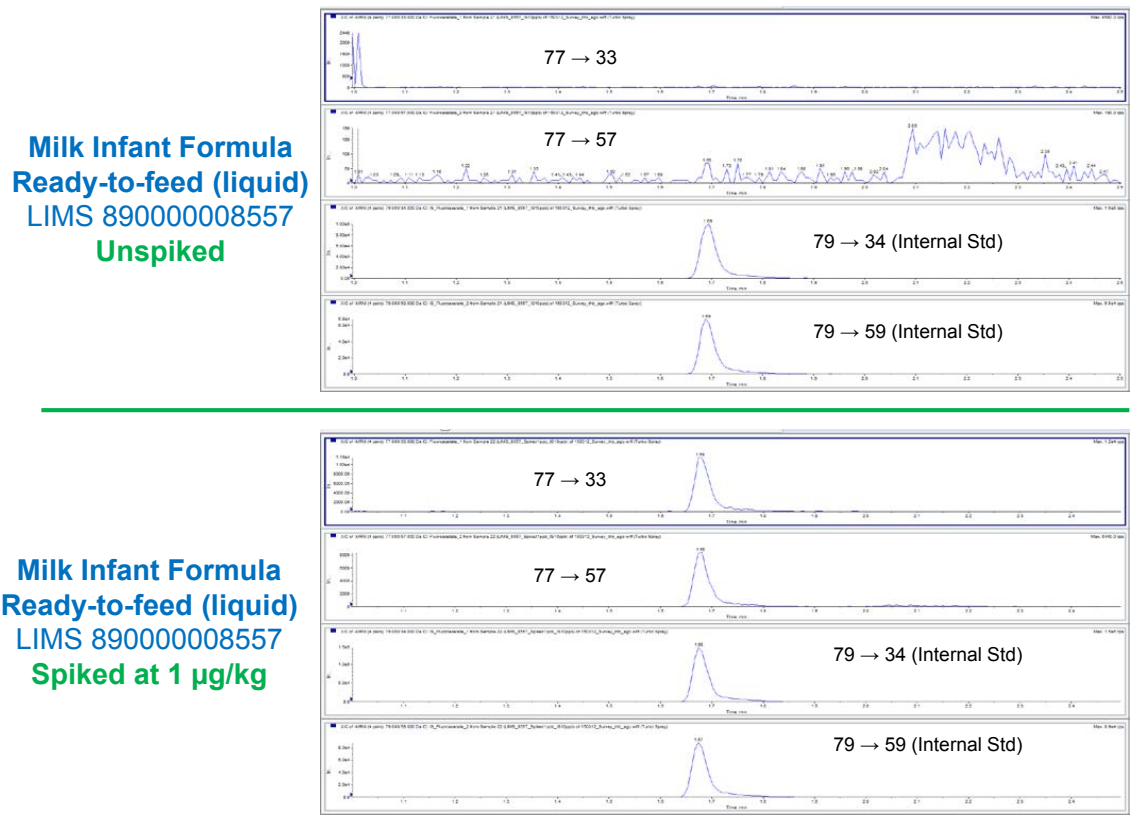
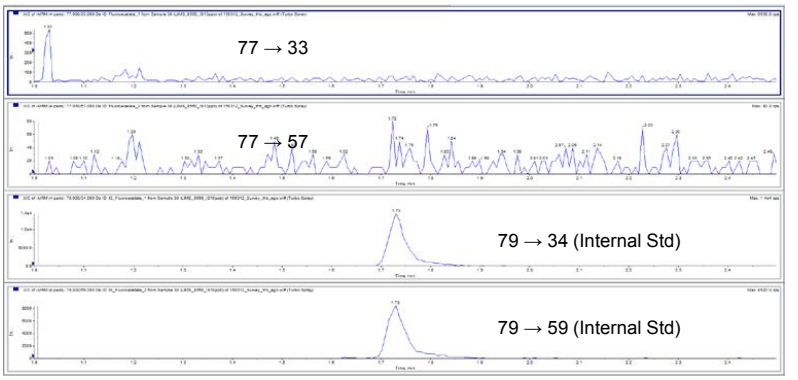


Figure 2015.03C. LC-MS/MS chromatograms of a liquid milk-based infant formula (ready-to-feed) unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Infant Formula
Soya-based
LIMS 890000008560
Unspiked



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

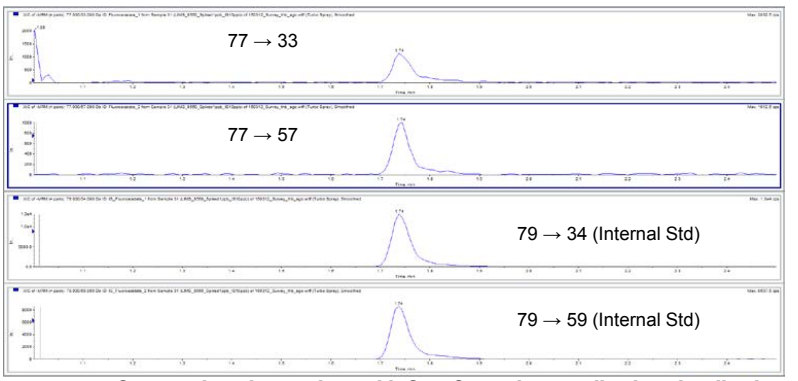
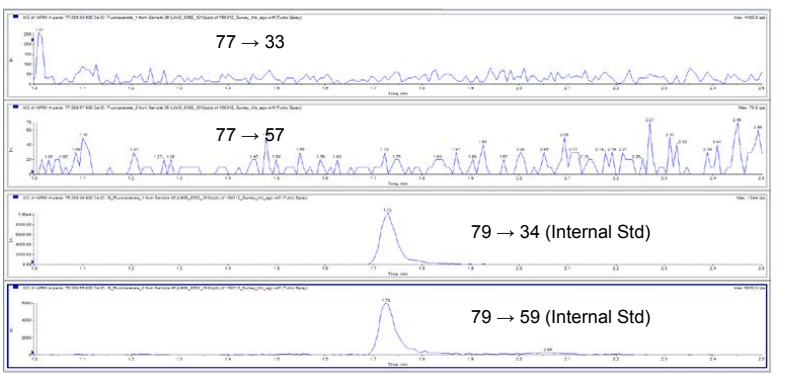


Figure 2015.03D. LC-MS/MS chromatograms of a powdered soya-based infant formula unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

Milk Infant Formula
LIMS 890000008562
Unspiked



Milk Infant Formula
LIMS 890000008562
Spiked at 1 µg/kg

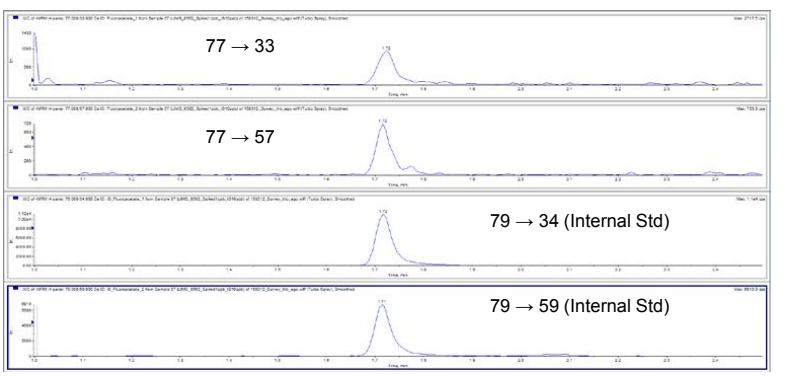


Figure 2015.03E. LC-MS/MS chromatograms of a powdered milk infant formula unspiked and spiked at the 1 µg/kg (IS 10 µg/kg) level.

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)

OFFICIAL METHODS

Determination of Monofluoroacetate in Powdered Nutritional Products by Derivatization with 2-Nitrophenylhydrazine and LC-MS/MS: First Action 2015.04

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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.

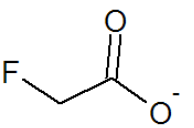

Figure 2015.04A. Monofluoroacetate $C_2H_2FO_2$, MW = 77.03, CAS No. 62-74-8.

(**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.

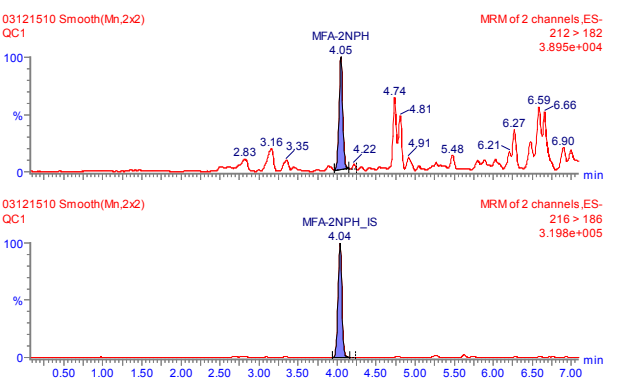

Figure 2015.04B. QC overspike at the method limit of quantitation (10 ng/g powder).

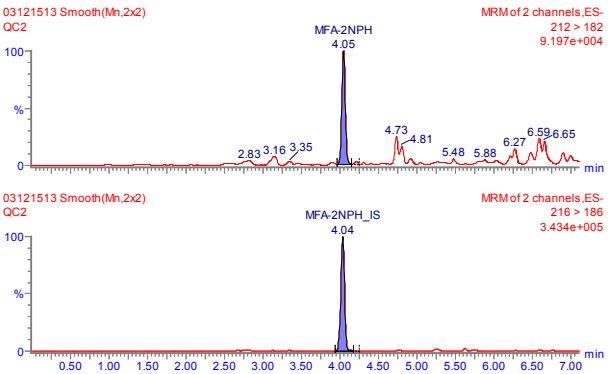

Figure 2015.04C. QC overspike Low at 25 ng/g powder.

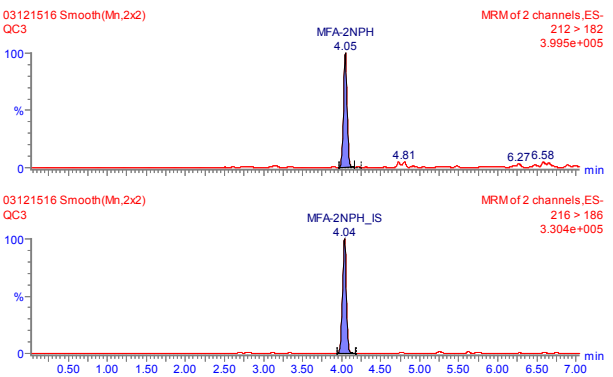

Figure 2015.04D. QC overspike Med at 100 ng/g powder.

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)

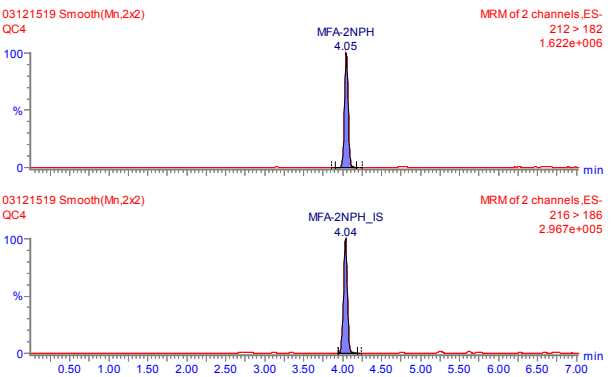

Figure 2015.04E. QC overspike High at 500 ng/g powder.

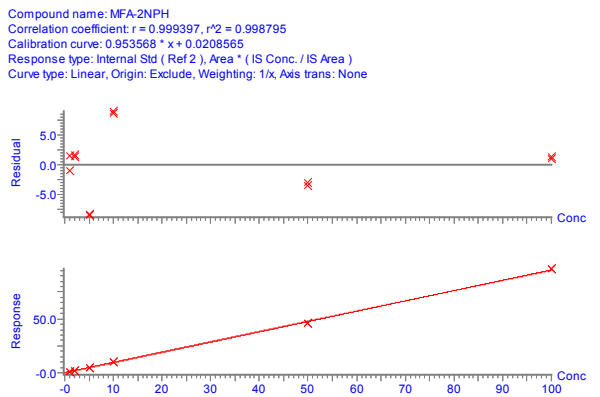

Figure 2015.04F. Calibration curve and residual plot.