AOAC INTERNATIONAL

Official Methods Board

Methods Reviewed by Official Methods Board (2015)

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2016 AOAC OFFICIAL METHODS BOARD AWARDS

CANDIDATES REVIEWED FOR

2015 METHOD OF THE YEAR

OFFICIAL METHODS OF ANALYSIS OF AOAC INTERNATIONAL

OMB AWARD CRITERIA USED FOR 2015 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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AOAC Official Method 2014.10
Dietary Starch
in Animal Feeds and Pet Food
Enzymatic-Colorimetric Method
First Action 2014

(Applicable for the determination of dietary starch in forages, grains, grain by-products, dry, semi-moist, and moist pet food products, and mixed feeds that range in concentration from 1 to 100%.)

Caution: Acetic acid is flammable in both liquid and vapor forms. It can cause severe skin burns and eye damage and is toxic if inhaled. Avoid breathing fumes. Wear protective gloves, clothing, and eye and face protection.

α-Amylase and glucose oxidase are respiratory sensitizers, which may cause allergy or asthma symptoms. Avoid breathing dust. Amylase preparations can cause allergic reactions in hypersensitive individuals. Avoid inhaling aerosols or dusts.

Benzoic acid causes serious eye damage and respiratory irritation. Avoid breathing dust and mist. Wear eye protection.

Phenol can be toxic and cause severe burns and eye damage. It is suspected of causing genetic defects and may cause damage to organs. Do not breathe dust or fumes. Wear protective gloves, clothing, eye protection, and face protection.

See Table **2014.10** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Ground or homogenized animal feed and pet food test portions are mixed with acetic acid buffer and heat-stable α-amylase and incubated at 100°C for 1 h with periodic mixing to gelatinize and partially hydrolyze the starch. After cooling, amyloglucosidase is added and the test mixture is incubated for 2 h at 50°C. The digested mixture is clarified, diluted as needed, and glucose detected in the resulting test solution using a colorimetric glucose oxidase-peroxidase (GOPOD) method that is sensitive and specific to glucose. Free glucose is measured simultaneously or in a separate analytical run for each test sample by carrying a second test portion of each test sample through the procedure omitting enzymes and incubating at 100°C for 1 h with periodic mixing. Dietary starch is determined as 0.9 times the difference of glucose in the digested test portion minus free glucose in the undigested test portion.

B. Apparatus

(**a**) *Grinding mill.*—Mills such as an abrasion mill equipped with a 1.0 to 0.5 mm screen, or a cutting mill with 0.5 mm screen, or other appropriate device to grind test samples to pass a 40 mesh screen.

(**b**) *Homogenizer, blender, or mixer.*—To provide homogenous suspension of canned pet food, liquid animal feed, semi-moist pet food, and other materials containing less than 85% dry matter.

(**c**) *Bench centrifuge or microcentrifuge.*—Capable of centrifuging at 1000 × *g* to 10 000 × *g*.

(**d**) *Water bath.*—Capable of maintaining 50 ± 1°C.

(**e**) *Vortex mixer.*

(**f**) *pH meter.*

(**g**) *Stop clock timer (digital).*

(**h**) *Top-loading balance.*—Capable of weighing accurately to ±0.01 g.

(**i**) *Analytical balance.*—Capable of weighing accurately to ±0.0001 g.

(**j**) *Laboratory ovens.*—With forced-convection; capable of maintaining 100 ± 1°C for carrying out incubations.

(**k**) *Spectrophotometer.*—Capable of operating at absorbances of 505 nm.

(**l**) *Pipets.*—Capable of delivering 0.1 and 1.0 mL; with disposable tips.

(**m**) *Positive-displacement repeating pipet.*—Capable of accurately delivering 0.1, 1.0, and 3.0 mL.

(**n**) *Dispenser.*—1000 mL or greater capacity; capable of accurately delivering 20 and 30 mL.

(**o**) *Glass test tubes.*—16 × 100 mm.

(**p**) *Glass tubes.*—25 × 200 mm, with polytetrafluoroethylene (PTFE)-lined screw caps or comparable tubes to hold 51.1 mL and allow for adequate mixing when sealed.

(**q**) *Plastic film.*—Or similarly nonreactive material.

(**r**) *Magnetic stir plate.*

(**s**) *Glass fiber filter.*—With 1.6 µm retention.

Table 2014.10. Method performance for determination of dietary starch in feeds

Material	No. of labs	Mean, %	s_r	s_R	RSD_r %	RSD_R %	r[a]	R[b]
Moist canned dog food	11	1.54	0.03	0.09	2.21	5.99	0.10	0.26
Low starch horse feed	13	7.02	0.23	0.36	3.32	5.19	0.65	1.02
Dry ground corn	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54
Complete dairy feed	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48
Soybean meal	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31
Distillers grains	13	4.11	0.11	0.20	2.67	4.94	0.31	0.57
Pelleted poultry feed	13	28.24	0.73	1.34	2.58	4.76	2.04	3.76
Corn silage	13	39.04	0.80	1.88	2.05	4.82	2.24	5.27
Dog kibble, dry	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46
Alfalfa pellets	13	1.38	0.12	0.13	8.61	9.69	0.33	0.38

[a] $r = 2.8 \times s_r$.

[b] $R = 2.8 \times s_R$.

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(**t**) *Hardened filter paper.*—With 22 µm retention.

C. Reagents

Note: Use high-quality distilled or deionized water for all water additions.

(**a**) *Acetate buffer (100 mM, pH 5.0).*—Weigh 6.0 g or pipet 5.71 mL glacial acetic acid and transfer immediately to a flask; quantitatively transfer weighed acid with H_2O rinses. Bring volume to ca 850 mL. While stirring solution on a magnetic stir plate, adjust pH to 5.0 ± 0.1 with 1 M NaOH solution. Dilute to 1 L with H_2O. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**b**) *Heat-stable α-amylase solution.*—Liquid, heat-stable, α-amylase (examples: Product Termamyl 120 L, Novozymes North America, Franklinton, NC, USA; Product Multifect AA 21L, Genencor International, Rochester, NY, USA; origin: *Bacillus licheniformis*, or equivalent). Should not contain greater than 0.5% glucose. pH optima must include 5.5–5.8.

Based on Bacterial Amylase Unit (BAU) method.—Approximately 83000 BAU/mL of concentrated enzyme (1 BAU is defined as the amount of enzyme that will dextrinize starch at the rate of 1 mg/min at pH 6.6 and 30 ± 0.1°C; 1). If modifications of volume delivered are necessary due to enzymatic activity of the enzyme used, the volume used per test portion should deliver approximately 8300 ± 20 BAU (1).

The enzymes should be of a purity meeting the specifications listed in **991.43** (*see* 32.1.17), but as modified below for application in the assay for dietary starch. The enzyme preparation used must be validated within laboratory to verify efficacy, as well as lack of interference. Recommended validation: analyze 0.1 g test portions of purified glucose, sucrose, and purified corn starch with the enzymatic portion of the dietary starch assay and using a free glucose value of zero in calculations. Analyses with candidate enzyme should give values of [mean ± standard deviation (SD)] glucose: 90 ± 2%, starch: 100±2%, and sucrose: 0.7 ± 0.3% on a dry matter basis. To test for interference from release of glucose from fiber carbohydrates, analyze 0.1 g test portions of α-cellulose and barley β-glucan that are not contaminated with free glucose with the enzymatic portion of the dietary starch assay. Recovery of these substrates should be less than 0.5% on a dry matter basis [*see* **991.43** (*see* 32.1.17)]. Use AOAC approved methods for determination of dry matters of the samples. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%), or background absorbance readings will interfere with test sample measurements.

(**c**) *Diluted amyloglucosidase solution.*—Dilute concentrated amyloglucosidase with 100 mM sodium acetate buffer, **C**(**a**), to give 1 mL of solution per test portion with 2 to 5 mL excess. Add 1/3 of needed buffer to an appropriately sized graduated cylinder. Pipet concentrated amyloglucosidase into buffer, rinsing tip by taking up and expelling buffer in the graduated cylinder. Bring to desired volume with additional buffer. Cap cylinder with plastic film and invert cylinder repeatedly to mix. The concentrated amyloglucosidase used should not contain greater than 0.5% glucose, and should have a pH optimum of 4.0 and pH stability between 4.0–5.5 (example of concentrated amyloglucosidase: Product E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland; origin: *Aspergillus niger*, or equivalent).

(*1*) *Based on release of glucose from soluble starch or glycogen.*—200 U/mL (1 unit of enzyme activity is defined as the amount of enzyme required to release 1 µmole glucose/min at pH 4.5 and 40°C; 21).

(*2*) *Based on p-nitrophenyl-β-maltoside method.*—13 units/mL (1 unit is defined as the amount of enzyme required to release 1 µmole *p*-nitrophenol from *p*-nitrophenyl-β-maltoside/min at pH 4.5 and 40°C; 2). Follow the protocol described in **C**(**b**) for standards and procedure for testing adequacy of enzyme activity and lack of side activity.

The enzyme used must be validated within laboratory to verify efficacy as well as lack of interference. Use the same validation procedure as described for heat-stable α-amylase, **C**(**b**).

(**d**) *Benzoic acid solution (0.2%).*—Weigh 2.0 g benzoic acid (solid, ACS reagent, >99.5% purity) and add to a flask. Bring flask to 1 L volume with H_2O. Add magnetic stir bar, stopper flask, and allow to stir overnight to dissolve benzoic acid. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**e**) *GOPOD reagent.*—(*1*) *Mixture of glucose oxidase, 7000 U/L, free from catalase activity; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74 mM.*—Prepare by dissolving 9.1 g Na_2HPO_4 (dibasic, anhydrous) and 5.0 g KH_2PO_4 in ca 300 mL H_2O in a 1 L volumetric flask. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add 1.0 g phenol (ACS grade) and 0.15 g 4-aminoantipyrine. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add glucose oxidase (7000 U) and peroxidase (7000 U), rinse enzymes into flask with H_2O, and swirl gently to dissolve without causing excessive foaming. Bring to 1 L volume with H_2O. Seal and invert repeatedly to mix. Filter solution through a glass fiber filter with 1.6 µm retention, **B**(**s**). Store in a sealed amber bottle at ca 4°C. Reagent life: 1 month. Before use in test sample determinations, determine a standard curve for the reagent using a 5-point standard curve using **C**(**e**) and **C**(**f**) according to **D**(**b**).

(*2*) Alternatively, use another AOAC-approved glucose-specific assay that has passed in laboratory validation to accurately determine glucose concentrations of glucose standard solutions and give values equivalent to the values listed for determination of efficacy of enzymes. Recommended validation: analyze all five glucose working standard solutions and 100 mg test portions of purified glucose, purified sucrose, and purified corn starch that have been processed through the enzymatic hydrolysis portion of the dietary starch procedure and using a free glucose value of zero in calculations. The glucose values of the working standard solutions should be predicted ±6 µg glucose/mL. On a dry matter basis, the control sample glucose should give a dietary starch value (mean ± SD) of 90 ± 2%, corn starch at 100 ± 2%, and sucrose 0.7 ± 0.3%.

(**f**) *Glucose working standard solutions.*—0, 250, 500, 750, and 1000 µg/mL. Determine the dry matter of powdered crystalline glucose (purity ≥99.5%) by an AOAC-approved method. Weigh approximately 62.5, 125, 187.5, and 250 mg portions of glucose and record weight to 0.0001 g. Rinse each portion of glucose from weigh paper into a separate 250 mL volumetric flask with 0.2% benzoic acid solution, **C**(**d**), and swirl to dissolve. Bring each standard to 250 mL volume with 0.2% benzoic acid solution, **C**(**d**), to give four independent glucose standard solutions. The 0.2% benzoic acid solution, **C**(**d**), serves as the 0 µg/mL standard solution. Multiply weight of glucose by dry matter percentage and percentage purity as provided by the manufacturer in the certificate of analysis and divide by 250 mL to calculate actual glucose concentrations of the solutions. Prepare solutions at least one day before use to allow

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equilibration of α- and β- forms of the glucose. Standard solutions may be stored at room temperature for 6 months.

(**g**) *Internal quality control samples*.—Powdered crystalline glucose (purity ≥99.5%) and isolated corn starch. For the corn starch sample, crude protein as nitrogen content × 6.25 and ash should be determined to determine the nonprotein organic matter content of the sample. For use in recovery calculations, actual starch content of the corn starch control sample is estimated as 100% minus ash% and minus crude protein%, all on a dry matter basis. Analyze 100 mg of each sample with each batch of test samples. Glucose will allow evaluation of quantitative recovery, and starch will allow evaluation of quantitative recovery and efficacy of the assay.

(**h**) *Determination of accuracy of volume additions for use of summative volume approach*.—The method as described relies on accurate volumetric additions in order to use the sum of volumes to describe test solution volume. Accuracy of volume additions can be evaluated before the assay by the following procedure: Using 1–2 L distilled water at ambient temperature, determine the g/mL density of the water by recording the weight of three empty volumetric flasks (volumes between 50 and 100 mL), add the water to bring to volume, and weigh the flasks + water. Calculate water density g/mL as:

$$\text{Water density, g/mL} = [(\text{flask + water, g}) - (\text{flask, g})]/\text{water volume mL}$$

Record the weights of five empty tubes used for the dietary starch assay. Using the ambient temperature water and the devices used to deliver the liquid volumes for the enzymatic hydrolysis portion of the assay, deliver the 30, 0.1, 1, and 20 mL volumes to each tube (total of 51.1 mL in each tube). Record the weight of each tube + water. Calculate the grams of water in each tube as:

$$\text{Water in each tube, g} = (\text{tube + water, g}) - (\text{tube, g})$$

Divide the weight of water in each tube by the determined average density of water to give the volume of water in each tube. The deviation should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. If the deviations are greater than these, after the addition of 20 mL water during the dietary starch assay, individual samples should be quantitatively transferred with filtration through hardened filter paper with a 22 µm retention, **B**(**t**), into a 100 mL volumetric flask and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

D. Preparation of Reagent Blanks, Standard Curves, and Test Samples

(**a**) *Reagent blank*.—For each assay, two reaction tubes containing only the reagents added for each method are carried through the entire procedure. Reagent blanks diluted to the same degree as samples (no dilution or diluted to the same degree as control and test samples) are analyzed. Absorbance values for the reagent blanks are subtracted from absorbance values of the test solutions prepared from test and control samples.

(**b**) *Standard curves*.—Pipet 0.1 mL of 0.2% benzoic acid solution, **C**(**d**), and nominal 250, 500, 750, and 1000 µg/mL working standard glucose solutions, **C**(**f**), in duplicate into the bottoms of 16 × 100 mm glass culture tubes. Add 3.0 mL GOPOD reagent, **C**(**e**), to each tube using a positive displacement repeating pipet aimed against wall of tube, so it will mix well with the sample. Vortex tubes. Cover tops of tubes with plastic film. Incubate in a 50°C water bath for 20 min. Read absorbance at 505 nm using the 0 µg glucose/mL standard to zero the spectrophotometer. All readings should be completed within 30 min of the end of incubation; avoid subjecting solutions to sunlight as this degrades the chromogen. Calculate the quadratic equation describing the relationship of glucose µg/mL (response variable) and absorbance (abs) at 505 nm (independent variable) using all individual absorbances (do not average within standard). The equation will have the form:

$$\text{Glucose, µg/mL} = \text{abs} \times \text{quadratic coefficient} + \text{abs} \times \text{linear coefficient} + \text{intercept}$$

Use this standard curve to calculate glucose µg/mL in test solutions. A new standard curve should be run with each glucose determination run.

(**c**) *Test samples*.—Feed and pet food amenable to drying should be dried at 55°C in a forced-air oven. Dried materials are then ground to pass the 0.5 or 1.0 mm screen of an abrasion mill or the 0.5 mm screen of a cutting mill or other mill to give an equivalent fineness of grind (to pass a 40 mesh screen). Ground, dried materials are transferred into a wide mouthed jar and mixed well by inversion and tumbling before subsampling. Semi-moist, moist, or liquid products may be homogenized, blended, or mixed to ensure homogeneity and reduced particle size (3).

E. Determination of Dietary Starch

The analyses for free glucose and enzymatically released glucose + free glucose may be performed in separate analytical runs. For flow of assay, *see* Figure **2014.10**.

(*1*) Accurately weigh two test portions (W_E, W_F) of 100 to 500 mg each of dried test samples or 500 mg semi-moist, moist, or liquid samples (for all samples, use ≤500 mg, containing

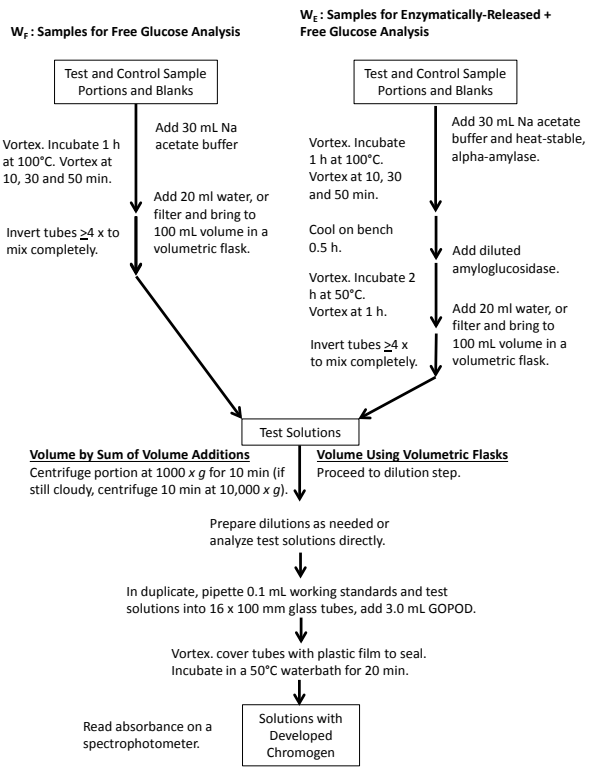

Figure 2014.10. Flow chart of the dietary starch assay.

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≤100 mg dietary starch; use 500 mg for samples containing <2% dietary starch) into screw-cap glass tubes. Test portion W_E is for the analysis of enzymatically released glucose and W_F is for the determination of free glucose. In addition to unknowns, weigh test portions (W_E, W_F) of D-glucose and purified corn starch, which serve as quality control samples **C**(**g**). Also include two tubes with no test portion to serve as reagent blanks per each analytical run for free glucose or enzymatically released glucose + free glucose.

(*2*) Dispense 30 mL of 0.1 M sodium acetate buffer, **C**(**a**), into each tube.

(*3*) To tubes with test portions designated W_E and to each of the reagent blanks to be used with analysis of enzymatically released glucose + free glucose, add a volume of heat-stable, α-amylase, **C**(**b**), to deliver ca 1800 to 2100 liquefon units or 8200 to 8300 BAU of enzyme activity (typically 0.1 mL enzyme as purchased); do not add the amylase to W_F and to the reagent blanks to be used with free glucose determinations. Cap tubes and vortex to mix.

Note: Vortex tube so that the solution column extends to the cap, washing the entire interior of the tube and dispersing the test portion.

(*4*) Incubate all tubes for 1 h at 100°C in a forced-air oven, vortexing tubes at 10, 30, and 50 min of incubation.

(*5*) Cool tubes at ambient temperature on bench for 0.5 h. At this point, separate tubes designated for free glucose analysis (tubes containing W_F test portions and reagent blanks with no enzyme) from the rest of the run. Those designated for free glucose should skip steps (*6*) and (*7*) and continue with steps (*8*)–(*13*).

(*6*) Add 1 mL of diluted amyloglucosidase solution, **C**(**c**), to W_E test and quality control samples and reagent blanks. Vortex tubes.

(*7*) Incubate tubes for 2 h in a water bath at 50°C, vortexing at 1 h of incubation.

(*8*) Add 20 mL water to each tube. Cap and invert at least four times to mix completely. Proceed immediately through steps (*9*)–(*13*).

(*9*) (*a*) *Volume by sum of volume additions*.—Transfer ca 1.5 mL test sample solutions to microcentrifuge tubes, and centrifuge at 1000 × *g* for 10 min. If the sample remains cloudy after centrifugation, centrifuge an additional 10 min at 10 000 × *g* to clarify the solution before proceeding. Solutions may increase in temperature during centrifugation; allow centrifuged solutions to come to room temperature before preparing dilution.

(*b*) *Volume using volumetric flasks*.—Quantitatively transfer test sample solutions with filtration through a hardened paper filter with 22 µm retention and rinses with water to 100 mL volumetric flasks.

(*10*) Prepare dilutions as needed with distilled or deionized water. Solutions from control samples and test samples estimated to give greater than 1000 µg glucose/mL concentrations of free and released glucose should be diluted 1 in 10 if processed as in (*9*)(*a*) or 1 in 5 if processed as in (*9*)(*b*). Reagent blanks should be diluted to provide solutions with the same dilutions as used with the test solutions, so that the diluted reagent blank solutions can be used to make corrections for similarly diluted test solutions. Dilutions may be prepared using volumetric flasks or by accurate pipetting. If done by pipetting, use a minimum of 0.5 mL test sample or control solution to minimize the impact of variation in pipetting small volumes.

(*11*) Pipet 0.1 mL in duplicate of glucose working standard solutions (0, 250, 500, 750, and 1000 µg/mL glucose), **C**(**f**), and reagent blank, quality control sample, and test sample solutions into the bottoms of 16 × 100 mm glass test tubes using two tubes/solution. Add 3.0 mL GOPOD reagent, **C**(**e**)(*1*), to each tube. Vortex tubes. Place tubes in a rack and cover with plastic film to seal.

Note: Alternative to the use of the GOPOD method, proceed with alternate glucose determination method, **C**(**e**)(*2*), for measurement of glucose in working standards, reagent blank, control sample, and test sample solutions.

(*12*) Incubate in a 50°C water bath for 20 min.

(*13*) Set spectrophotometer to measure absorbance at 505 nm. After the incubation is complete, zero the spectrophotometer with the GOPOD-reacted 0 µg/mL working standard solution. Read absorbances of remaining GOPOD-reacted working standard solutions, and reagent blank, control sample, and test sample solutions. All reacted solutions must be read within 30 min of the end of the GOPOD incubation. The duplicate absorbance values are averaged for each reagent blank, test sample, and control sample solution and used in *Calculations*.

F. Calculations

Determine the quadratic equation that fits the absorbances of the working standard solutions. The absorbance values, A_{CF} or A_{CE}, are the independent variables (X), and actual glucose concentrations are the dependent variables (Y). Individual absorbance values of the working standard solutions, not averages, are used. The equation has the form:

$$\mu g\ Glucose/mL = (A_{CF\ or\ CE}^{2} \times Q + A_{CF\ or\ CE} \times S + I)$$

Calculate dietary starch content in test sample as received as follows:

$$\text{Free glucose, \%} = (A_{CF}^{2} \times Q + A_{CF} \times S + I) \times V_F \times DF_F \times 1/1\,000\,000 \times 1/W_F \times 162/180 \times 100$$

$$\text{Dietary starch, \%} = [(A_{CE}^{2} \times Q + A_{CE} \times S + I) \times V_E \times DF_E \times 1/1\,000\,000 \times 1/W_E \times 162/180 \times 100] - \text{free glucose \%}$$

where subscript F represents values for samples analyzed for free glucose and subscript E represents values for samples treated with amylase and amyloglucosidase; A_{CF}, A_{CE} = absorbance of reaction solutions minus the absorbance of the appropriately diluted reagent blank, values are averages of the two replicates for each test solution; Q = quadratic slope term, S = linear slope term, and I = intercept of the standard curve to convert absorbance values to µg glucose/mL; V_F, V_E = final sample solution volume, ca 50.0 mL for V_F and 51.1 mL for V_E if done by summation of volumetric additions, otherwise, by size of volumetric flask used; DF = dilution factor, e.g., 0.5 mL sample solution diluted into 5.0 mL = 5.0/0.5 = 10; 1 g/1 000 000 µg = conversion from µg to g; W_E, W_F = test portion weight, as received; 162/180 = factor to convert from measured glucose as determined, to anhydroglucose, as occurs in starch.

If test samples are run in duplicate portions, the free glucose % in the dietary starch equation is the average free glucose % value determined for the test sample.

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Candidates for 2016 Method of the Year

FOOD COMPOSITION AND ADDITIVES

Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method: Collaborative Study

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Starch, glycogen, maltooligosaccharides, and other α-1,4- and α-1,6-linked glucose carbohydrates, exclusive of resistant starch, are collectively termed "dietary starch". This nutritionally important fraction is increasingly measured for use in diet formulation for animals as it can have positive or negative effects on animal performance and health by affecting energy supply, glycemic index, and formation of fermentation products by gut microbes. AOAC Method 920.40 that was used for measuring dietary starch in animal feeds was invalidated due to discontinued production of a required enzyme. As a replacement, an enzymatic-colorimetric starch assay developed in 1997 that had advantages in ease of sample handling and accuracy compared to other methods was considered. The assay was further modified to improve utilization of laboratory resources and reduce time required for the assay. The assay is quasi-empirical: glucose is the analyte detected, but its release is determined by run conditions and specification of enzymes. The modified assay was tested in an AOAC collaborative study to evaluate its accuracy and reliability for determination of dietary starch in animal feedstuffs and pet foods. In the assay, samples are incubated in screw cap tubes with thermostable α-amylase in pH 5.0 sodium acetate buffer for 1 h at 100°C with periodic mixing to gelatinize and partially hydrolyze α-glucan. Amyloglucosidase is added, and the reaction mixture is incubated at 50°C for 2 h and mixed once. After subsequent addition of water, mixing, clarification, and dilution as needed, free + enzymatically released glucose are measured. Values from a separate determination of free glucose are subtracted to give values for enzymatically released glucose. Dietary starch equals enzymatically released glucose multiplied by 162/180 (or 0.9) divided by the weight of the as received sample. Fifteen laboratories that represented feed company, regulatory, research, and commercial feed testing laboratories analyzed 10 homogenous test materials representing animal feedstuffs and pet foods in duplicate using the dietary starch assay. The test samples ranged from 1 to 70% in dietary starch content and included moist canned dog food, alfalfa pellets, distillers grains, ground corn grain, poultry feed, low starch horse feed, dry dog kibbles, complete dairy cattle feed, soybean meal, and corn silage. The average within-laboratory repeatability SD (s_r) for percentage dietary starch in the test samples was 0.49 with a range of 0.03 to 1.56, and among-laboratory repeatability SDs (s_R) averaged 0.96 with a range of 0.09 to 2.69. The HorRat averaged 2.0 for all test samples and 1.9 for test samples containing greater than 2% dietary starch. The HorRat results are comparable to those found for AOAC Method 996.11, which measures starch in cereal products. It is recommended that the dietary starch method be accepted for Official First Action status.

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The method was approved by the Expert Review Panel for Dietary Starch.

The Expert Review Panel for Dietary Starch 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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Starch is an important, frequently analyzed component of animal feedstuffs. It can have substantial positive effects on animal performance and potential undesirable effects on glycemic response and animal health (1). AOAC *Official Method*℠ **920.40** for starch in animal feeds (2) is no longer valid because of discontinued production of the enzyme "Rhozyme-S" (Rohm and Haas, Philadelphia, PA) specified in the procedure. Accordingly, another approved method for starch in animal feeds is needed. Additionally, new terminology is needed to define "starch" to more accurately describe the nutritionally relevant fraction of interest, and the definition can be used to specify the analysis.

Starch has long been defined as a natural vegetable polymer consisting of long linear unbranched chains of α-1,4-linked D-glucose units (amylose) and/or long α-1,6-branched chains of α-1,4-linked glucose units (amylopectin; 3). However, the amylases and amyloglucosidases that specifically hydrolyze the linkages in plant starch also hydrolyze those same linkages in glycogen from animal (4) or microbial (5) sources and in maltooligosaccharides that are breakdown products of starch

but are not polysaccharides. Accordingly, enzymatic starch methods do not measure plant starch alone (6), unless animal and microbial ingredients and the feedstuffs that contain them are excluded from analysis. From a nutritional standpoint, inclusion of glycogen, starch, and maltooligosaccharides more completely describes the pool of carbohydrate that is potentially available to digestion by salivary or small intestinal amylases or amyloglucosidases (7), but the pool can not be called "starch" because that term is well established as referring to a plant polysaccharide.

Recognizing the aim of nutritional characterization, the Laboratory Methods & Services Committee of the Association of American Feed Control Officials with involvement of researchers and industry arrived at a definition for "Dietary Starch": An alpha-linked-glucose carbohydrate of or derived from plants, animals, or microbes from which glucose is released through the hydrolytic actions of purified α-amylases and amyloglucosidases that are specifically active only on α-(1-4) and α-(1-6) linkages in feed materials that have been gelatinized in heated, mildly acidic buffer. Its concentration in feed is determined by enzymatically converting the α-linked glucose carbohydrate to glucose and then measuring the liberated glucose. This definition encompasses plant starch, glycogen, maltooligosaccharides, and maltose/isomaltose. The use of mildly acidic buffer for the gelatinization excludes the use of alkali or dimethyl sulfoxide and, thus, excludes resistant starch from inclusion in the dietary starch fraction.

The proposed dietary starch method avoids known analytical defects and allows handling of diverse physical forms of samples. It is based on an assay published by Bach Knudsen (8) that was slightly modified to improve use of laboratory resources, reduce run time, and maintain starch recovery (9). It is similar in chemistry to AOAC Method **996.11** (10), but differs in the buffer used and in sample handling procedures and gave a greater recovery of starch (9). Specific to the dietary starch assay, all enzymatic reactions are carried out in an acidic buffer that improves recovery by limiting the production of maltulose, an isomerization product produced at more neutral pH (11). Maltulose is resistant to enzymatic hydrolysis and reduces starch recovery. The use of a screw cap tube as a reaction vessel allows for more vigorous mixing, which is useful for all types of feed materials but may be essential for those that clump, are moist, or do not behave like dry, ground powders. Although enzymes used in development of the method will be listed, learning from the loss of AOAC Method **920.40** (2), this assay will not be set to use specific commercial enzymes but rather enzymes with specific activity that give desired results under the conditions of the method. The detection method specified is a colorimetric glucose oxidase-peroxidase method based on an assay developed by Karkalas (12), but recommendations are made to use other approved chromatographic analyses if interferences such as antioxidants are present.

Collaborative Study

Method Performance Parameters and Optimization

The performance parameters of the dietary starch procedure were investigated by the Study Director, who developed the method evaluated in this study. The following factors were evaluated:

(*1*) *Repeatability.*—As tested previously in a single laboratory, the SDs of within laboratory replicates for dietary starch analysis of food and feed substrates were low (dietary starch mean = 46.9%, s_r = 0.48%; dry matter basis; 9).

(*2*) *LOD.*—LOD for the dietary starch assay was calculated from absorbance values as the mean reagent blank value + 3 × SD (13). The means and SD were calculated for the absorbances of duplicate readings for seven undiluted with-enzyme reagent blanks from six separate assay runs. For each reagent blank, the value of the mean absorbance + 3 SD was used in the glucose standard curve determined for that run to calculate the detected glucose value. This value was multiplied by the final reaction volume (51.1 mL), by 162/180 to convert glucose to a starch basis, and converted to g. The calculated dietary starch LOD are 0.3% of sample weight based on analysis of a 100 mg test portion.

(*3*) *Accuracy/recovery.*—Recovery of pure corn starch was determined on samples analyzed singly in five separate analytical runs and in duplicate in an additional run. The average recovery ± SD was 99.3 ± 0.8% on a dry matter basis. In the collaborative study, the average dietary starch value for the control corn starch sample was 89.9 ± 3.7% on an as received basis with an estimated actual value of 89.4%.

(*4*) *Linearity.*—Linearity of the dietary starch assay was evaluated on a dry matter basis using purified corn starch samples weighing 25, 50, 75, and 100 mg analyzed on 3 separate days. The effect of starch amount tended to have a linear effect on recovery (P = 0.07), but the difference was small at a maximum of 2 percentage units between the highest and lowest recoveries. The least squares means ± SD for recovery were 101.9 ± 1.7, 99.9 ± 0.2, 100.3 ± 0.4, and 100.0 ± 0.7% for 25, 50, 75, and 100 mg of corn starch, respectively.

(*5*) *Specificity.*—The dietary starch method gave very low values (mean ± SD) for sucrose (0.17 ± 0.00% of sample dry matter), α-cellulose (0.03 ± .02% of air dried sample), and isolated oat beta-glucan (0.31 ± 0.09% of air dried sample), indicating that run conditions and enzyme preparations used did not appreciably hydrolyze these feed components. Sucrose, in particular, has been shown to interfere with starch analysis (14), likely due to side activity of the enzyme preparations used. Use of separate free glucose determinations allows correction for free glucose and background absorbance associated with each sample. The final detection method, the glucose oxidase – peroxidase (GOPOD) method, is specific for glucose, which limits interference from other carbohydrates.

(*6*) *Interference.*—Antioxidants can depress glucose detection in the GOPOD assay. Addition of ascorbic acid as a model antioxidant gave a linear decrease in absorbance at additions of greater than 10 μmoles of ascorbic acid (15). The effect was relatively small up to 10 μmol of ascorbic acid. Investigations into the antioxidant content of foodstuffs (16) showed that most of the high starch or leafy vegetable foods had hydrophilic antioxidant values that would be equivalent to less than 10 μmoles of ascorbic acid/0.1 g of dry matter. Exceptions included foods high in phenolic compounds (e.g., beets and red sorghum grain with antioxidant content approximately equivalent to 23 and 14 μmol ascorbic acid, respectively). Because of the interference in the GOPOD assay, another method for measuring glucose should be considered for feeds or foods exceeding 10 to 20 μmol of hydrophilic antioxidant/0.1 g of test sample dry matter.

(*7*) *Use of quadratic standard curves.*—The standard curves in the GOPOD assay are slightly nonlinear, and this is normal for this assay within the glucose concentrations commonly used (15). The linear equations describing glucose standard curves had R^2 of nearly 1.0 (0.9998 to 1.0) suggesting a very good fit to the linear form but the intercepts were not 0. Thus, when the standard curves were used to predict glucose concentrations of the standard solutions used to produce them, the predicted values frequently differed slightly from the expected values. It was determined that a quadratic form fit the standard curves better than a linear form based on significance of the quadratic term in the regression equation, the reduction in the root mean squared error of the standard curve and the relative decrease in residual sums of squares (residual = observed minus predicted) between the linear and quadratic equations, and evaluation of the residual versus predicted value plots (15). Other nonlinear forms were not explored.

(*8*) *Determination of final volume by summation of liquid additions.*—The method uses summing of added reagent volumes or use of volumetric flasks to give the final volume of test solutions before dilution. Total volumes of test solutions and dilutions can be determined by summing of added volumes if accurately quantitative volumetric pipets and dispensers are used to add reagents. An evaluation of summation of volumes and determination of final volume by weight and density showed no difference in recovery of glucose ($P = 0.21$) or of corn starch ($P = 0.62$) analyzed with the dietary starch assay. The density of test sample solutions and reagent blanks appears to be quite consistent (0.999 g/mL, SD = 0.002, $n = 120$ from 16 analysis runs over 16 months). Accuracy of reagent additions can be determined by the final weight of total added liquid [(weight of tube + test sample + liquid) minus (weight of tube + test sample)]. The weights of total added liquid are 49.9 and 51.0 g for the portions of the assay run without or with enzyme additions, respectively. The deviations from these values should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. Alternatively, after the addition of water, test solutions can be quantitatively transferred with filtration through Whatman (Florham Park, NJ) 54 or equivalent paper into 100 mL volumetric flasks and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

(*9*) *Ease of use/efficiency.*—The method has the advantage that all reagent additions are made to samples in tubes that can be handled in racks. It does not require transfer of sample until the final dilution and measurement of glucose. Vortexing of the sealed tubes rinses the entire interior of the tube with solution, thus minimizing the possibility that test samples will escape contact with reagents. Studies verified the acceptability of using the same temperature for the amyloglucosidase digestion and glucose analysis incubations (15), which allowed more economic use of laboratory resources.

(*10*) *Use of control samples.*—The use of glucose and corn starch as control samples allows evaluation of quantitative recovery, and starch allows evaluation of quantitative recovery and efficacy of the assay.

(*11*) *Evaluation of enzymes for suitability.*—It is essential that the enzymes and run conditions used release only glucose bound by α-1,6- and α-1,4-linkages and give close to 100% recovery of corn starch. Sucrose is the most common interfering carbohydrate encountered in feedstuffs (14) typically due to its hydrolysis through side activity of the enzyme preparations used. Though the run conditions used will not hydrolyze sucrose, commonly available enzyme preparations have activity that can and are thus unsuitable for this assay. Analysis of glucose, corn starch, and sucrose with candidate enzymes should give values (mean ± SD) of glucose 90 ± 2%, starch 100 ± 2%, and sucrose 0.7 ± 0.3% on a dry matter basis. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%) or background absorbance readings will interfere with test sample measurements.

(*12*) *Method of glucose detection.*—The dietary starch protocol specifies use of an enzymatic-colorimetric assay that has been found to be very precise (15). However, it also allows use of other AOAC-approved glucose-specific assays that have been proven in laboratory validation to be appropriate for the dietary starch assay. On this basis, qualifying assays that are devoid of interference and are, thus, more suitable for use on specific matrixes, or are preferred in a given laboratory may be used.

Collaborating Laboratories

The 15 laboratories that participated in the study represented eight regulatory laboratories, three commercial feed testing laboratories, two feed company laboratories, and two research laboratories. One each of research, commercial feed testing, and regulatory laboratories that expressed interest in participating did not complete the study. Participating laboratories received no compensation. Collaborators were provided with blind test samples, control glucose and corn starch, thermostable α-amylase (Multifect AA 21L, Genencor International, Rochester, NY), amyloglucosidase (E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland), glucose standards, electronic data sheets, and larger reaction tubes if needed. They were required to prepare the GOPOD reagent, perform the dietary starch assay as written, analyze test samples in duplicate, and provide comments and detailed result forms containing both raw and calculated data describing their analyses of three blind familiarization test materials, 10 blind collaborative study test materials, and control samples for dietary starch.

Materials

Test materials selected for the collaborative study covered a wide range of dietary starch contents, ranging from 1 to 69% on an as-received basis and derived from single batches of manufactured and commodity feedstuffs used with different animal species. The test sample grinding and homogenizing methods used were designed to produce materials that would pass a 40 mesh screen. By virtue of their diverse handling characteristics, a number of different methods were used to prepare the samples for analysis. Corn silage, poultry feed, low starch horse feed, and alfalfa pellets were ground through the 6 mm screen of a cutting mill (Pulverisette 19, Fritsch GmbH, Idar-Oberstein, Germany) and then processed through the 0.5 mm screen of a centrifugal mill (ZM200 with 12 blade knife, Retsch GmbH, Haan, Germany). Dry corn, soybean meal, and distillers grains were ground to pass the 0.5 mm screen of a centrifugal mill (ZM200 with 12 blade knife), as

Table 1. Homogeneity of dietary starch for four sample sets of each test material[a]

Material	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R,%	2.8 × s_r	2.8 × s_R	HorRat
Moist canned dog food	4	1.58	0.01	0.02	0.86	1.06	0.04	0.05	0.29
Low starch horse feed	4	7.17	0.06	0.11	0.85	1.56	0.17	0.31	0.53
Dry ground corn	4	72.70	0.34	0.34	0.46	0.46	0.95	0.95	0.22
Complete dairy feed	4	28.38	0.10	0.40	0.34	1.40	0.27	1.11	0.58
Soybean meal	4	1.17	0.05	0.05	3.94	3.94	0.13	0.13	1.01
Distillers grains	4	4.23	0.06	0.06	1.37	1.37	0.16	0.16	0.43
Pelleted poultry feed	4	28.50	0.32	0.32	1.14	1.14	0.91	0.91	0.47
Corn silage	4	41.15	1.06	1.06	2.58	2.58	2.98	2.98	1.13
Dog kibble, dry	4	27.82	0.95	1.01	3.43	3.64	2.67	2.83	1.50
Alfalfa pellets	4	1.46	0.04	0.05	3.06	3.18	0.12	0.13	0.84

[a] s_r = SD of repeatability within sample; s_R = SD within and among sample sets; RSD_r = repeatability SD; RSD_R = reproducibility SD.

was the textured dairy complete feed but with dry ice used in the grinding of this sample. Dog kibble was ground with a kitchen processing mill (Assistent, MagicMill, Upper Saddle River, NJ) and further processed through a blending mill (1095 Knifetec sample mill, Foss Tecator, Höganäs, Sweden). The moist, canned dog food was homogenized with a commercial blender (Waring laboratory blender, 14-509-66, Fisher Scientific, Pittsburgh, PA). Dry ground test samples were subsampled using a rotary splitter (Laborette 27, Fritsch GmbH) and stored at –20°C in vacuum sealed bags (3.5 mil nylon polyethylene standard barrier vacuum bag, DCE, Inc., Springville, CA) until shipment. Homogenized moist dog food was transferred to individual sealed plastic bags (Whirl-Pak 58 mL, B01009WA, Nasco, Fort Atkinson, WI) and stored at –20°C. Test sample weights/bag were approximately 20 g for dried ground samples and 25 g of homogenized moist dog food.

For the collaborative study, individual test samples were labeled with a letter. Dry test samples and control samples were packed together in a sealed plastic bag. The homogenized moist dog food test sample and enzymes were packaged in an insulated container with a frozen ice pack. Materials were shipped overnight to the laboratories with directions to place the homogenized moist dog food test sample in the freezer until analysis. That sample was to be thawed overnight at 4°C, and all analyses in the dietary starch procedure were to be performed on it on the following day; no such limitations were placed on analyses of the dry test samples.

As per the example of Mertens (17), dietary starch analyses in duplicate of four randomly selected samples of each test material were used to evaluate random variation within and among samples. In this application, the SD of repeatability within sample (s_r) and SD of reproducibility among laboratories (s_R) calculated using the AOAC spreadsheet designed for evaluating collaborative studies represent the variation within and between separate samples of test materials as tested in the Study Director's laboratory. The s_r and s_R were similar within each sample, indicating that the prepared test samples were homogenous (Table 1). The HorRat values for corn silage and dog kibble were greater than 1.1. As concluded in a similar evaluation (17), these results suggest that these samples were less homogenous or for some reason more difficult to analyze for dietary starch than the other samples. For the dog kibble test sample, small dark particles that did not dissolve or degrade and had the coloration of one form of kibble present in the original unground material were visible in the acetate buffer during incubations.

Statistical Analyses

Data from all laboratories were reviewed for data entry and calculation errors before statistical evaluation, and results were reverified if values were identified as outliers. Ranking scores (18) were used to identify laboratories that were outliers across all materials. Data from the one such identified laboratory were excluded from further data analysis.

The AOAC INTERNATIONAL Interlaboratory Study Workbook for Evaluation of Blind Duplicates (Version 2.0, 2006) spreadsheet was used to evaluate data from the collaborative study and from the homogeneity test performed in the Study Director's laboratory.

AOAC Official Method 2014.10
Dietary Starch in Animal Feeds and Pet Food
Enzymatic-Colorimetric Method
First Action 2014

(Applicable for the determination of dietary starch in forages, grains, grain by-products, dry, semi-moist, and moist pet food products, and mixed feeds that range in concentration from 1 to 100%.)

Caution: Acetic acid is flammable in both liquid and vapor forms. It can cause severe skin burns and eye damage and is toxic if inhaled. Avoid breathing fumes. Wear protective gloves, clothing, and eye and face protection.

α-Amylase and glucose oxidase are respiratory sensitizers, which may cause allergy or asthma symptoms. Avoid breathing dust. Amylase preparations can cause allergic reactions in hypersensitive individuals. Avoid inhaling aerosols or dusts.

Benzoic acid causes serious eye damage and respiratory irritation. Avoid breathing dust and mist. Wear eye protection.

Phenol can be toxic and cause severe burns and eye damage. It is suspected of causing genetic defects and may cause damage to organs. Do not breathe dust or fumes. Wear protective gloves, clothing, eye protection, and face protection.

See Table **2014.10** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Ground or homogenized animal feed and pet food test portions are mixed with acetic acid buffer and heat-stable α-amylase and incubated at 100°C for 1 h with periodic mixing to gelatinize and partially hydrolyze the starch. After cooling, amyloglucosidase is added and the test mixture is incubated for 2 h at 50°C. The digested mixture is clarified, diluted as needed, and glucose detected in the resulting test solution using a colorimetric glucose oxidase-peroxidase (GOPOD) method that is sensitive and specific to glucose. Free glucose is measured simultaneously or in a separate analytical run for each test sample by carrying a second test portion of each test sample through the procedure omitting enzymes and incubating at 100°C for 1 h with periodic mixing. Dietary starch is determined as 0.9 times the difference of glucose in the digested test portion minus free glucose in the undigested test portion.

B. Apparatus

(**a**) *Grinding mill.*—Mills such as an abrasion mill equipped with a 1.0 to 0.5 mm screen, or a cutting mill with 0.5 mm screen, or other appropriate device to grind test samples to pass a 40 mesh screen.

(**b**) *Homogenizer, blender, or mixer.*—To provide homogenous suspension of canned pet food, liquid animal feed, semi-moist pet food, and other materials containing less than 85% dry matter.

(**c**) *Bench centrifuge or microcentrifuge.*—Capable of centrifuging at 1000 × *g* to 10 000 × *g*.

(**d**) *Water bath.*—Capable of maintaining 50 ± 1°C.

(**e**) *Vortex mixer.*

(**f**) *pH meter.*

(**g**) *Stop clock timer (digital).*

(**h**) *Top-loading balance.*—Capable of weighing accurately to ±0.01 g.

(**i**) *Analytical balance.*—Capable of weighing accurately to ±0.0001 g.

(**j**) *Laboratory ovens.*—With forced-convection; capable of maintaining 100 ± 1°C for carrying out incubations.

(**k**) *Spectrophotometer.*—Capable of operating at absorbances of 505 nm.

(**l**) *Pipets.*—Capable of delivering 0.1 and 1.0 mL; with disposable tips.

(**m**) *Positive-displacement repeating pipet.*—Capable of accurately delivering 0.1, 1.0, and 3.0 mL.

(**n**) *Dispenser.*—1000 mL or greater capacity; capable of accurately delivering 20 and 30 mL.

(**o**) *Glass test tubes.*—16 × 100 mm.

(**p**) *Glass tubes.*—25 × 200 mm, with polytetrafluoroethylene (PTFE)-lined screw caps or comparable tubes to hold 51.1 mL and allow for adequate mixing when sealed.

(**q**) *Plastic film.*—Or similarly nonreactive material.

(**r**) *Magnetic stir plate.*

(**s**) *Glass fiber filter.*—With 1.6 µm retention.

(**t**) *Hardened filter paper.*—With 22 µm retention.

C. Reagents

Note: Use high-quality distilled or deionized water for all water additions.

(**a**) *Acetate buffer (100 mM, pH 5.0).*—Weigh 6.0 g or pipet 5.71 mL glacial acetic acid and transfer immediately to a flask; quantitatively transfer weighed acid with H_2O rinses. Bring volume to ca 850 mL. While stirring solution on a magnetic stir plate, adjust pH to 5.0 ± 0.1 with 1 M NaOH solution. Dilute to 1 L with H_2O. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**b**) Heat-stable α-amylase solution.—Liquid, heat-stable, α-amylase (examples: Product Termamyl 120 L, Novozymes North America, Franklinton, NC; Product Multifect AA 21L, Genencor International, Rochester, NY; origin: Bacillus licheniformis, or equivalent). Should not contain greater than 0.5% glucose. pH optima must include 5.5–5.8.

Based on Bacterial Amylase Unit (BAU) method.—Approximately 83000 BAU/mL of concentrated enzyme (1 BAU is defined as the amount of enzyme that will dextrinize starch at the rate of 1 mg/min at pH 6.6 and 30 ± 0.1°C; 19). If modifications of volume delivered are necessary due to enzymatic activity of the enzyme used, the volume used per test portion should deliver approximately 8300 ± 20 BAU (19).

Table 2014.10. Method performance for determination of dietary starch in feeds

Material	No. of labs	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	r^a	R^b
Moist canned dog food	11	1.54	0.03	0.09	2.21	5.99	0.10	0.26
Low starch horse feed	13	7.02	0.23	0.36	3.32	5.19	0.65	1.02
Dry ground corn	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54
Complete dairy feed	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48
Soybean meal	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31
Distillers grains	13	4.11	0.11	0.20	2.67	4.94	0.31	0.57
Pelleted poultry feed	13	28.24	0.73	1.34	2.58	4.76	2.04	3.76
Corn silage	13	39.04	0.80	1.88	2.05	4.82	2.24	5.27
Dog kibble, dry	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46
Alfalfa pellets	13	1.38	0.12	0.13	8.61	9.69	0.33	0.38

[a] $r = 2.8 \times s_r$

[b] $R = 2.8 \times s_R$.

The enzymes should be of a purity meeting the specifications listed in *Official Method*SM **991.43** (20), but as modified below for application in the assay for dietary starch. The enzyme preparation used must be validated within laboratory to verify efficacy, as well as lack of interference. Recommended validation: analyze 0.1 g test portions of purified glucose, sucrose, and purified corn starch with the enzymatic portion of the dietary starch assay and using a free glucose value of zero in calculations. Analyses with candidate enzyme should give values of [mean ± standard deviation (SD)] glucose: 90 ± 2%, starch: 100±2%, and sucrose: 0.7 ± 0.3% on a dry matter basis. To test for interference from release of glucose from fiber carbohydrates, analyze 0.1 g test portions of α-cellulose and barley β-glucan that are not contaminated with free glucose with the enzymatic portion of the dietary starch assay. Recovery of these substrates should be less than 0.5% on a dry matter basis (20). Use AOAC approved methods for determination of dry matters of the samples. Enzyme preparations must not contain appreciable concentrations of glucose (<0.5%), or background absorbance readings will interfere with test sample measurements.

(**c**) *Diluted amyloglucosidase solution.*—Dilute concentrated amyloglucosidase with 100 mM sodium acetate buffer, **C**(**a**), to give 1 mL of solution per test portion with 2 to 5 mL excess. Add 1/3 of needed buffer to an appropriately sized graduated cylinder. Pipet concentrated amyloglucosidase into buffer, rinsing tip by taking up and expelling buffer in the graduated cylinder. Bring to desired volume with additional buffer. Cap cylinder with plastic film and invert cylinder repeatedly to mix. The concentrated amyloglucosidase used should not contain greater than 0.5% glucose, and should have a pH optimum of 4.0 and pH stability between 4.0–5.5 (example of concentrated amyloglucosidase: Product E-AMGDF, Megazyme International Ireland, Ltd., Bray, Co. Wicklow, Ireland; origin: *Aspergillus niger*, or equivalent).

(*1*) *Based on release of glucose from soluble starch or glycogen.*—200 U/mL (1 unit of enzyme activity is defined as the amount of enzyme required to release 1 µmole glucose/min at pH 4.5 and 40°C; 21).

(*2*) *Based on p-nitrophenyl-β-maltoside method.*—13 units/mL (1 unit is defined as the amount of enzyme required to release 1 µmole *p*-nitrophenol from *p*-nitrophenyl-β-maltoside/min at pH 4.5 and 40°C; 22).

The enzyme used must be validated within laboratory to verify efficacy as well as lack of interference. Use the same validation procedure as described for heat-stable α-amylase, **C**(**b**).

(**d**) *Benzoic acid solution (0.2%).*—Weigh 2.0 g benzoic acid (solid, ACS reagent, >99.5% purity) and add to a flask. Bring flask to 1 L volume with H_2O. Add magnetic stir bar, stopper flask, and allow to stir overnight to dissolve benzoic acid. This can be done in an Erlenmeyer flask or beaker that has been made volumetric by weighing or transferring 1 L water into the vessel and then etching the meniscus line for the known volume.

(**e**) *GOPOD reagent.*—(*1*) *Mixture of glucose oxidase, 7000 U/L, free from catalase activity; peroxidase, 7000 U/L; and 4-aminoantipyrine, 0.74 mM.*—Prepare by dissolving 9.1 g Na_2HPO_4 (dibasic, anhydrous) and 5.0 g KH_2PO_4 in ca 300 mL H_2O in a 1 L volumetric flask. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add 1.0 g phenol (ACS grade) and 0.15 g 4-aminoantipyrine. Use H_2O to rinse chemicals into bulb of flask. Swirl to dissolve completely. Add glucose oxidase (7000 U) and peroxidase (7000 U), rinse enzymes into flask with H_2O, and swirl gently to dissolve without causing excessive foaming. Bring to 1 L volume with H_2O. Seal and invert repeatedly to mix. Filter solution through a glass fiber filter with 1.6 µm retention, **B**(**s**). Store in a sealed amber bottle at ca 4°C. Reagent life: 1 month. Before use in test sample determinations, determine a standard curve for the reagent using a 5-point standard curve using **C**(**e**) and **C**(**f**) according to **D**(**b**).

(*2*) Alternatively, use another AOAC-approved glucose-specific assay that has passed in laboratory validation to accurately determine glucose concentrations of glucose standard solutions and give values equivalent to the values listed for determination of efficacy of enzymes. Recommended validation: analyze all five glucose working standard solutions and 100 mg test portions of purified glucose, purified sucrose, and purified corn starch that have been processed through the enzymatic hydrolysis portion of the dietary starch procedure and using a free glucose value of zero in calculations. The glucose values of the working standard solutions should be predicted ±6 µg glucose/mL. On a dry matter basis, the control sample glucose should give a dietary starch value (mean ± SD) of 90 ± 2%, corn starch at 100 ± 2%, and sucrose 0.7 ± 0.3%.

(**f**) *Glucose working standard solutions.*—0, 250, 500, 750, and 1000 µg/mL. Determine the dry matter of powdered crystalline glucose (purity ≥99.5%) by an AOAC-approved method. Weigh approximately 62.5, 125, 187.5, and 250 mg portions of glucose and record weight to 0.0001 g. Rinse each portion of glucose from weigh paper into a separate 250 mL volumetric flask with 0.2% benzoic acid solution, **C**(**d**), and swirl to dissolve. Bring each standard to 250 mL volume with 0.2% benzoic acid solution, **C**(**d**), to give four independent glucose standard solutions. The 0.2% benzoic acid solution, **C**(**d**), serves as the 0 µg/mL standard solution. Multiply weight of glucose by dry matter percentage and percentage purity as provided by the manufacturer in the certificate of analysis and divide by 250 mL to calculate actual glucose concentrations of the solutions. Prepare solutions at least one day before use to allow equilibration of α- and β- forms of the glucose. Standard solutions may be stored at room temperature for 6 months.

(**g**) *Internal quality control samples*.—Powdered crystalline glucose (purity ≥99.5%) and isolated corn starch. For the corn starch sample, crude protein as nitrogen content × 6.25 and ash should be determined to determine the nonprotein organic matter content of the sample. For use in recovery calculations, actual starch content of the corn starch control sample is estimated as 100% minus ash% and minus crude protein%, all on a dry matter basis. Analyze 100 mg of each sample with each batch of test samples. Glucose will allow evaluation of quantitative recovery, and starch will allow evaluation of quantitative recovery and efficacy of the assay.

(**h**) *Determination of accuracy of volume additions for use of summative volume approach*.—The method as described relies on accurate volumetric additions in order to use the sum of volumes to describe test solution volume. Accuracy of volume additions can be evaluated before the assay by the following procedure: Using 1–2 L distilled water at ambient temperature, determine the g/mL density of the water by recording the weight of three empty volumetric flasks (volumes between 50 and

100 mL), add the water to bring to volume, and weigh the flasks + water. Calculate water density g/mL as:

$$\text{Water density g/mL} = [(\text{flask + water, g}) - (\text{flask, g})]/\text{water volume mL}$$

Record the weights of five empty tubes used for the dietary starch assay. Using the ambient temperature water and the devices used to deliver the liquid volumes for the enzymatic hydrolysis portion of the assay, deliver the 30, 0.1, 1, and 20 mL volumes to each tube (total of 51.1 mL in each tube). Record the weight of each tube + water. Calculate the grams of water in each tube as:

$$\text{Water in each tube, g} = (\text{tube + water, g}) - (\text{tube, g})$$

Divide the weight of water in each tube by the determined average density of water to give the volume of water in each tube. The deviation should be no more than 0.5% or 0.25 g on average, or 1.0% or 0.5 g for any individual tube for the summative volume addition approach to be used. If the deviations are greater than these, after the addition of 20 mL water during the dietary starch assay, individual samples should be quantitatively transferred with filtration through hardened filter paper with a 22 µm retention, **B**(**t**), into a 100 mL volumetric flask and brought to volume to fix the sample solution volume before clarification, dilution, and analysis.

D. Preparation of Reagent Blanks, Standard Curves, and Test Samples

(**a**) *Reagent blank.*—For each assay, two reaction tubes containing only the reagents added for each method are carried through the entire procedure. Reagent blanks diluted to the same degree as samples (no dilution or diluted to the same degree as control and test samples) are analyzed. Absorbance values for the reagent blanks are subtracted from absorbance values of the test solutions prepared from test and control samples.

(**b**) *Standard curves.*—Pipet 0.1 mL of 0.2% benzoic acid solution, **C**(**d**), and nominal 250, 500, 750, and 1000 µg/mL working standard glucose solutions, **C**(**f**), in duplicate into the bottoms of 16 × 100 mm glass culture tubes. Add 3.0 mL GOPOD reagent, **C**(**e**), to each tube using a positive displacement repeating pipet aimed against wall of tube, so it will mix well with the sample. Vortex tubes. Cover tops of tubes with plastic film. Incubate in a 50°C water bath for 20 min. Read absorbance at 505 nm using the 0 µg glucose/mL standard to zero the spectrophotometer. All readings should be completed within 30 min of the end of incubation; avoid subjecting solutions to sunlight as this degrades the chromogen. Calculate the quadratic equation describing the relationship of glucose µg/mL (response variable) and absorbance (abs) at 505 nm (independent variable) using all individual absorbances (do not average within standard). The equation will have the form:

$$\text{Glucose, µg/mL} = \text{abs x quadratic coefficient} + \text{abs} \times \text{linear coefficient} + \text{intercept}$$

Use this standard curve to calculate glucose µg/mL in test solutions. A new standard curve should be run with each glucose determination run.

(**c**) *Test samples.*—Feed and pet food amenable to drying should be dried at 55°C in a forced-air oven. Dried materials are then ground to pass the 0.5 or 1.0 mm screen of an abrasion mill or the 0.5 mm screen of a cutting mill or other mill to give an equivalent fineness of grind (to pass a 40 mesh screen). Ground, dried materials are transferred into a wide mouthed jar and mixed well by inversion and tumbling before subsampling. Semi-moist, moist, or liquid products may be homogenized, blended, or mixed to ensure homogeneity and reduced particle size (23).

E. Determination of Dietary Starch

The analyses for free glucose and enzymatically released glucose + free glucose may be performed in separate analytical runs. For flow of assay, *see* Figure **2014.10**.

(*1*) Accurately weigh two test portions (W_E, W_F) of 100 to 500 mg each of dried test samples or 500 mg semi-moist, moist, or liquid samples (for all samples, use ≤500 mg, containing ≤100 mg dietary starch; use 500 mg for samples containing <2% dietary starch) into screw-cap glass tubes. Test portion W_E is for the analysis of enzymatically released glucose and W_F is for the determination of free glucose. In addition to unknowns, weigh test portions (W_E, W_F) of D-glucose and purified corn starch, which serve as quality control samples **C**(**g**). Also include two tubes with no test portion to serve as reagent blanks per each analytical run for free glucose or enzymatically released glucose + free glucose.

(*2*) Dispense 30 mL of 0.1 M sodium acetate buffer, **C**(**a**), into each tube.

(*3*) To tubes with test portions designated W_E and to each of the reagent blanks to be used with analysis of enzymatically released glucose + free glucose, add a volume of heat-stable, α-amylase, **C**(**b**), to deliver ca 1800 to 2100 liquefon units or 8200 to 8300 BAU of enzyme activity (typically 0.1 mL enzyme as purchased); do not add the amylase to W_F and to the reagent blanks to be used with free glucose determinations. Cap tubes and vortex to mix.

Note: Vortex tube so that the solution column extends to the cap, washing the entire interior of the tube and dispersing the test portion.

(*4*) Incubate all tubes for 1 h at 100°C in a forced-air oven, vortexing tubes at 10, 30, and 50 min of incubation.

(*5*) Cool tubes at ambient temperature on bench for 0.5 h. At this point, separate tubes designated for free glucose analysis (tubes containing W_F test portions and reagent blanks with no enzyme) from the rest of the run. Those designated for free glucose should skip steps (*6*) and (*7*) and continue with steps (*8*)–(*13*).

(*6*) Add 1 mL of diluted amyloglucosidase solution, **C**(**c**), to W_E test and quality control samples and reagent blanks. Vortex tubes.

(*7*) Incubate tubes for 2 h in a water bath at 50°C, vortexing at 1 h of incubation.

(*8*) Add 20 mL water to each tube. Cap and invert at least 4 times to mix completely. Proceed immediately through steps (*9*)–(*13*).

(*9*) (*a*) *Volume by sum of volume additions.*—Transfer ca 1.5 mL test sample solutions to microcentrifuge tubes, and centrifuge at 1000 x *g* for 10 min. If the sample remains cloudy after centrifugation, centrifuge an additional 10 min at

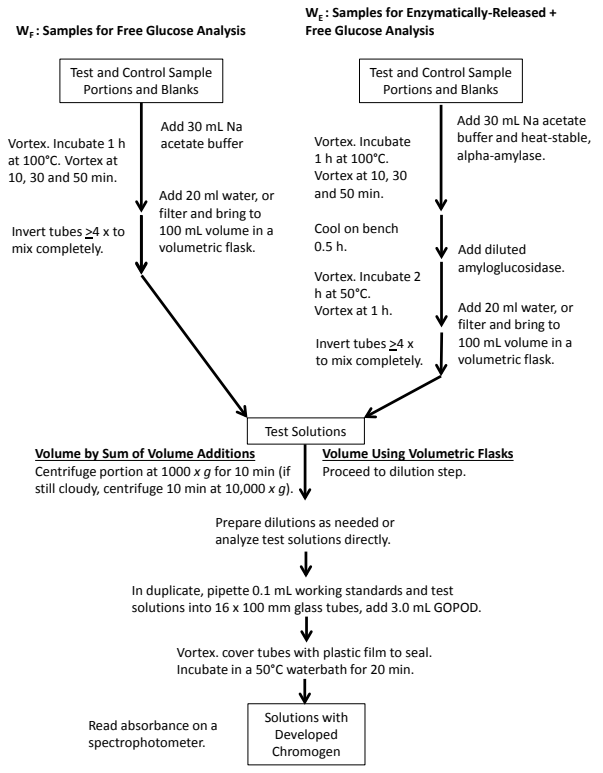

Figure 2014.10. Flow chart of the dietary starch assay.

10 000 × *g* to clarify the solution before proceeding. Solutions may increase in temperature during centrifugation; allow centrifuged solutions to come to room temperature before preparing dilution.

(*b*) *Volume using volumetric flasks*.—Quantitatively transfer test sample solutions with filtration through a hardened paper filter with 22 µm retention and rinses with water to 100 mL volumetric flasks.

(*10*) Prepare dilutions as needed with distilled or deionized water. Solutions from control samples and test samples estimated to give greater than 1000 µg glucose/mL concentrations of free and released glucose should be diluted 1 in 10 if processed as in (*9*)(*a*) or 1 in 5 if processed as in (*9*)(*b*). Reagent blanks should be diluted to provide solutions with the same dilutions as used with the test solutions, so that the diluted reagent blank solutions can be used to make corrections for similarly diluted test solutions. Dilutions may be prepared using volumetric flasks or by accurate pipetting. If done by pipetting, use a minimum of 0.5 mL test sample or control solution to minimize the impact of variation in pipetting small volumes.

(*11*) Pipet 0.1 mL in duplicate of glucose working standard solutions (0, 250, 500, 750, and 1000 µg/mL glucose), **C**(**f**), and reagent blank, quality control sample, and test sample solutions into the bottoms of 16 × 100 mm glass test tubes using two tubes/solution. Add 3.0 mL GOPOD reagent, **C**(**e**)(*1*), to each tube. Vortex tubes. Place tubes in a rack and cover with plastic film to seal.

Note: Alternative to the use of the GOPOD method, proceed with alternate glucose determination method, **C**(**e**)(*2*), for measurement of glucose in working standards, reagent blank, control sample, and test sample solutions.

(*12*) Incubate in a 50°C water bath for 20 min.

(*13*) Set spectrophotometer to measure absorbance at 505 nm. After the incubation is complete, zero the spectrophotometer with the GOPOD-reacted 0 µg/mL working standard solution. Read absorbances of remaining GOPOD-reacted working standard solutions, and reagent blank, control sample, and test sample solutions. All reacted solutions must be read within 30 min of the end of the GOPOD incubation. The duplicate absorbance values are averaged for each reagent blank, test sample, and control sample solution and used in *Calculations*.

F. Calculations

Determine the quadratic equation that fits the absorbances of the working standard solutions. The absorbance values, A_{CF} or A_{CE}, are the independent variables (X), and actual glucose concentrations are the dependent variables (Y). Individual absorbance values of the working standard solutions, not averages, are used. The equation has the form:

$$\mu g\ Glucose/mL = (A_{CF\ or\ CE}^{2} \times Q + A_{CF\ or\ CE} \times S + I)$$

Calculate dietary starch content in test sample as received as follows:

$$\text{Free glucose, \%} = (A_{CF}^{2} \times Q + A_{CF} \times S + I) \times V_F \times DF_F \times 1/1\,000\,000 \times 1/W_F \times 162/180 \times 100$$

$$\text{Dietary starch, \%} = [(A_{CE}^{2} \times Q + A_{CE} \times S + I) \times V_E \times DF_E \times 1/1\,000\,000 \times 1/W_E \times 162/180 \times 100] - \text{free glucose \%}$$

where subscript *F* represents values for samples analyzed for free glucose and subscript *E* represents values for samples treated with amylase and amyloglucosidase; A_{CF}, A_{CE} = absorbance of reaction solutions minus the absorbance of the appropriately diluted reagent blank, values are averages of the two replicates for each test solution; Q = quadratic slope term, S = linear slope term, and I = intercept of the standard curve to convert absorbance values to µg glucose/mL; V_F, V_E = final sample solution volume, ca 50.0 mL for V_F and 51.1 mL for V_E if done by summation of volumetric additions, otherwise, by size of volumetric flask used; DF = dilution factor, e.g., 0.5 mL sample solution diluted into 5.0 mL = 5.0/0.5 = 10; 1 g/1 000 000 µg = conversion from µg to g; W_E, W_F = test portion weight, as received; 162/180 = factor to convert from measured glucose as determined, to anhydroglucose, as occurs in starch.

If test samples are run in duplicate portions, the free glucose % in the dietary starch equation is the average free glucose % value determined for the test sample.

Results and Discussion

Evaluation of the Dietary Starch Method

Initial evaluation of data from all laboratories showed that most outliers occurred in two laboratories (Table 2). Laboratory 14 had significant Cochran's tests for five of the test materials, indicating suspect replicate results within this laboratory. Unlike the other laboratories, Laboratory 14 ran duplicate portions of test materials on separate days, rather than together within the same run. Based on laboratory ranking scores (18), this laboratory was designated as an outlier and its data were

Table 2. Results of collaborating laboratories for dietary starch individual replicate values on an as-received basis

		Collaborating laboratory														
Material	Duplicate	0	1	2	3	4	5	6	7	8	9	10	11[a]	12	13	14[b]
Moist canned dog food	1	1.58	1.42	1.34	1.56	1.58	1.58	1.57	1.64	1.59	1.44	**2.47**[c]	**1.94**[d]	1.55	**1.84**[c]	**0.22**[c]
	2	1.57	1.46	1.36	1.67	1.62	1.59	1.47	1.62	1.60	1.44	**1.59**[c]	**1.94**[d]	1.53	**1.61**[c]	**0.32**[c]
Low starch horse feed	1	7.03	6.29	7.01	7.30	6.78	7.21	6.88	7.33	7.27	6.47	6.68	8.32	7.15	7.02	**5.76**[c]
	2	7.21	6.50	7.44	7.60	6.43	7.61	7.02	7.33	6.98	6.74	7.37	7.87	7.08	6.68	**6.50**[c]
Dry ground corn	1	70.80	63.08	71.80	**58.85**[c]	71.27	68.13	70.18	71.22	71.52	71.25	67.97	**5.84**[d]	70.39	68.98	**60.19**[c]
	2	69.24	63.14	72.89	**26.64**[c]	70.23	67.33	71.47	73.29	71.08	70.04	65.82	**5.93**[d]	70.53	68.74	**65.42**[c]
Complete dairy feed	1	29.19	26.86	28.53	28.90	26.88	28.27	28.39	29.33	29.07	27.59	**26.89**[c]	**37.21**[d]	28.41	25.42	27.85
	2	29.79	26.69	28.49	30.02	26.11	28.70	28.19	29.10	28.89	27.49	**30.90**[c]	**35.45**[d]	28.01	26.10	27.28
Soybean meal	1	1.01	1.04	**1.09**[c]	1.10	0.97	1.13	0.94	1.04	1.06	0.87	1.02	**2.35**[d]	0.82	1.00	**0.02**[c]
	2	1.03	1.11	**1.42**[c]	1.19	0.93	1.11	0.90	0.93	1.09	0.78	1.16	**2.38**[d]	0.84	1.02	**0.82**[c]
Distillers grains	1	4.02	3.90	4.23	4.27	4.05	4.55	4.05	4.16	3.99	4.10	3.81	**4.82**[e]	4.19	3.98	**3.16**[c]
	2	4.07	3.90	4.09	4.30	4.08	4.49	3.94	4.14	4.06	4.06	4.09	**4.85**[e]	4.58	3.79	**3.00**[c]
Poultry feed	1	28.67	28.12	28.57	28.71	26.47	27.99	27.44	29.59	28.78	27.67	27.9	26.50	29.07	25.06	27.51
	2	29.25	27.35	27.95	30.26	28.00	28.27	28.52	29.43	28.83	27.65	30.39	25.18	29.45	24.80	26.56
Corn silage	1	41.10	37.44	39.20	40.92	37.54	39.18	38.08	39.17	40.91	37.00	37.26	36.03	43.50	36.59	37.99
	2	40.34	36.84	39.02	41.59	37.71	38.58	37.65	39.83	40.22	37.34	40.23	35.72	41.31	36.40	36.55
Dog kibble, dry	1	29.87	25.50	24.58	27.73	29.23	27.53	27.37	24.10	27.32	**17.99**[f]	25.73	27.55	28.68	26.30	24.31
	2	27.92	26.45	27.52	24.21	26.57	27.33	25.64	28.00	25.19	**18.35**[f]	27.25	26.93	29.34	25.70	26.25
Alfalfa pellets	1	1.29	1.17	1.56	1.32	1.56	1.59	1.61	1.35	1.33	1.58	1.42	1.31	1.13	1.25	0.60[c]
	2	1.36	1.43	1.43	1.41	1.32	1.61	1.31	1.24	1.34	1.38	1.35	1.13	1.38	1.27	**1.01**[c]

[a] Data for this laboratory was omitted from analysis based on a 7% change in glucose standard absorbances between runs for detection of free glucose and free + enzymatically released glucose. When data were included, four of 10 samples were identified as outliers by the single Grubbs' test, and one by the double Grubbs' test.

[b] Outlier laboratory detected by laboratory ranking.

[c] Outlier detected by the Cochran's test.

[d] Outlier detected by the single Grubbs' test.

[e] Outlier detected by the double Grubbs' test.

[f] Data omitted from analysis because the large test portion used (0.5 g) exceeded the 100 mg α-glucan limit for this assay.

not used in calculation of the study statistics. Laboratory 11 had four outlier values detected by the single Grubbs' test, which would indicate that this laboratory's values for these test samples were substantially higher or lower than those generated by the other laboratories. The very low value for dry ground corn appeared to be a possible error in recording the dilution of the sample, but laboratory records indicated that that was not the case. The basis for the high values for dairy feed, soybean meal, and moist canned dog food was not immediately obvious. The distillers grains results for Laboratory 11 was designated as an outlier based on results of the double Grubbs' test.

Laboratory 11 was not designated as an outlier by the ranking procedure, but test material results were generally higher for this laboratory. A likely basis for the higher dietary starch values was that the absorbances of the glucose standards were lower in the analytical run with the test samples treated with enzyme than were those reported for two other standard curves run for the dietary starch assay in that laboratory. The decrease in absorbance was on the order of 0.029 to 0.089 for 500 and 1000 mg glucose/mL standard solutions. To put this in perspective, the difference in absorbance values between runs represents an almost 8% lower absorbance value for the 1000 mg glucose/mL standard in the assay with enzyme-treated test samples. Standard curves produced from lower absorbance values will give higher calculated glucose and dietary starch values if the absorbances of the test samples are not similarly depressed. Absorbance values for glucose standards are not expected to be identical among analytical runs. However, the glucose oxidase-peroxidase assay used tends to be very consistent. For example, in the Study Director's laboratory, eight glucose standard curves run with dietary starch assays on 4 separate days showed RSD values (SD/mean) of less than 0.8% for absorbance values determined across runs within glucose standard (Table 3). Data from 12 collaborating laboratories that provided absorbance data for more than one standard curve showed the RSD of the absorbances calculated for individual glucose standards and then averaged across all standards were less than 1% for five laboratories, less than 2% for eight,and more than 2% for four (Table 4). Replicate absorbance readings for glucose standards within analytical run showed overall good repeatability for all laboratories. Laboratory 14, which was excluded from the study based on a ranking test, had the

Table 3. Absorbance values for glucose standards analyzed in repeated runs and in the collaborative study

Repeated analyses of glucose standard solutions: values by standard[a]						
Glucose standard, µg/mL	Runs[b]	Mean[c]	SD[d]	CV%[d]	Minimum value	Maximum value
249.4	8	0.285	0.0020	0.69	0.282	0.289
499.4	8	0.568	0.0028	0.49	0.563	0.574
748.7	8	0.848	0.0031	0.36	0.841	0.852
998.7	8	1.125	0.0045	0.40	1.116	1.133
Collaborative study: means across standards of values calculated for individual standards						
Laboratory	Runs	Overall mean[e]	Mean SD[f]	Mean CV, %[g]	Replicate SD[h]	
Study Director	3	0.704	0.0023	0.35	0.001	
7	2	0.688	0.0031	0.46	0.002	
8	4	0.712	0.0040	0.62	0.003	
13	2	0.658	0.0034	0.68	0.003	
2	2	0.855	0.0068	0.79	0.007	
1	6	0.827	0.0083	1.41	0.004	
12	3	0.684	0.0092	1.47	0.004	
3	2	0.736	0.0073	1.49	0.005	
6	2	0.723	0.0121	1.56	0.009	
4	3	0.682	0.0143	2.22	0.008	
5	4	0.727	0.0160	2.28	0.007	
11	3	0.709	0.0287	3.55	0.009	
14	2	0.667	0.0531	8.78	0.004	

[a] Glucose standards were analyzed in the Study Director's laboratory in eight separate analytical runs for the dietary starch assay. Glucose standards were analyzed in duplicate in two separate runs/day on 4 days. Two separate batches of GOPOD reagent were each used for four runs. The same preparations of glucose standards were used for all eight runs.

[b] Number of separate analytical runs in which the glucose standards were analyzed in duplicate.

[c] The mean value of the 16 replicates for each glucose standard.

[d] SD = standard deviation; RSD = 100 × (SD/mean).

[e] Mean of all absorbance values generated by the laboratory.

[f] The mean of all SD of absorbance values calculated for individual glucose standards.

[g] The mean of all RSD of absorbance values calculated for individual glucose standards.

[h] The mean of all SD of absorbance values for replicate pairs of glucose standards.

largest average RSD for absorbances of the glucose standards. Given the good replication for duplicates in this laboratory, the large RSD reflects differences in glucose standard absorbances between analytical runs. The difference this variation would generate in the standard curves could explain the variation detected in test sample replicates for this laboratory, because test sample duplicates were analyzed singly in separate runs, each of which used a different standard curve. Laboratory 11 had the second highest average RSD for absorbances of the standards. Discussions with Laboratory 11 did not uncover the basis for the variation between analytical runs. The dietary starch assay relies on the soundness of the standard curves to give reliable results. For Laboratory 11, because the glucose standard results used with the enzyme-treated samples deviated from two other standard curves they performed, and because the lower absorbances gave a standard curve that appears to have inflated the dietary starch values, the data are suspect and has been omitted from the statistical analysis of this study.

It is important to control the run to run and between replicate variation in analysis of the glucose standards because of the impact these have on accuracy of results. This GOPOD glucose detection assay is highly sensitive to pipetting accuracy. Samples should be read within 30 min of the end of incubation with GOPOD. It is also recommended that the incubated GOPOD-reacted samples be kept out of sunlight as this can degrade the chromagen. In addition to evaluating standard curve data for obvious changes in response, it is recommended that for each batch of GOPOD a log be kept of absorbance data for glucose standards from all runs. Within a glucose standard, calculate the SD of all absorbances. The mean of these SDs across all standards should not be greater than 0.016. Even lower levels of variability in absorbances can be readily achieved with this assay.

Another factor that likely affected accuracy was exceeding the 100 mg of starch limit/test portion in the assay, which was the case for Laboratory 9 when dry dog kibble was analyzed using 0.5 g test portions. The resulting low dietary starch values were likely the result of the enzyme no longer being in the excess required for complete hydrolysis of the dietary starch.

Variability of results may also have been affected by the approach to sample dilution. Laboratory 3 used 0.1 mL of test sample solution and 0.9 mL of water to make a 1 in 10 dilution

Candidates for 2016 Method of the Year

Table 4. Statistical data for dietary starch results

Material	Outlier	*n*	Mean, %	s_r	s_R	RSD_r, %	RSD_R, %	2.8 × s_r	2.8 × s_R	HorRat	Largest within-lab variance	Largest average lab result	Smallest average lab result
Moist canned dog food	10, 13	11	1.53	0.03	0.09	2.21	5.99	0.10	0.26	1.60	0.01	1.63	1.35
Low starch horse feed		13	7.02	0.23	0.36	3.32	5.19	0.65	1.02	1.74	0.24	7.45	6.40
Dry ground corn	3	12	69.60	0.86	2.69	1.23	3.87	2.40	7.54	1.83	2.31	72.34	63.11
Complete dairy feed	10	12	28.10	0.37	1.24	1.30	4.42	1.02	3.48	1.83	0.64	29.49	25.76
Soybean meal	2	12	1.00	0.05	0.11	4.97	11.16	0.14	0.31	2.79	0.01	1.15	0.83
Distillers grains		13	4.11	0.11	0.20	2.67	4.94	0.31	0.57	1.53	0.08	4.52	3.88
Pelleted poultry feed		13	28.24	0.73	1.34	2.58	4.76	2.04	3.76	1.97	3.10	29.51	24.93
Corn silage		13	39.04	0.80	1.88	2.05	4.82	2.24	5.27	2.09	4.41	42.40	36.49
Dog kibble, dry	9	12	26.88	1.56	1.59	5.82	5.92	4.38	4.46	2.43	7.61	29.01	25.97
Alfalfa pellets		13	1.38	0.12	0.13	8.61	9.69	0.33	0.38	2.54	0.05	1.60	1.25

for the ground corn sample. Even with small differences in pipetted amounts, such an approach could result in the between duplicate difference noted for that sample. Test solutions from the enzymatic hydrolysis procedure can be "sticky", i.e., they do not pipet exactly like water, and require care to pipet accurately. If dilutions are made by pipetting, prewetting of pipet tips and use of larger volumes, such as 0.5 mL of test solution and 4.5 mL of water, are recommended.

The quantity of test material used also may have affected assay variability. Test samples with starch contents of less than 2% generally showed greater variability than test samples that contained more starch (Figure 1 and Table 4) in a pattern nearly identical to that described by Wehling and DeVries (24) for dietary fiber assays. However, among the low starch materials, the moist dog food had RSD values for repeatability and reproducibility that were approximately half those of soybean meal and alfalfa pellets (Table 4); these latter two samples also had the highest HorRat values in the study. In addition to being the only moist, homogenized sample, laboratories were directed to use 0.5 g of the moist dog food as compared to 0.1 g of other samples. The one case in which dietary starch values for the moist dog food were identified by the Cochran test as suspect replicates within laboratory was where Laboratory 10 reported values determined on 0.10 g test samples for this material (Table 2). In the collaborative study, the 0.1 g sample size was used for most samples to minimize the likelihood that the 100 mg limit of dietary starch/test portion would be exceeded, based on the laboratories' prestudy results with the assay; however, it also greatly reduced the concentration of glucose to be detected in low starch test samples. Final glucose concentrations of test sample solutions for 0.1 g enzyme-treated test portions of soybean meal and alfalfa pellets were 22 and 30 µg/mL, respectively as compared to 167 µg/mL for the moist dog food using 0.5 g test portions. These glucose concentrations of the low starch feeds equate to absorbance values of 0.035, 0.054, and 0.221, respectively, as determined in the Study Director's laboratory. Although the glucose detection assay is sensitive and precise, small variations in absorbances of test solutions with very low glucose concentrations will give more variability in calculated glucose values than the same amount of variation will with test solutions with higher glucose concentrations. This can result in greater within and between laboratory variability for low starch test samples for which smaller test portions are used. In the case of the dietary starch assay, as with gravimetric dietary fiber analyses, the increase in RSD as concentrations of the analyte approaches zero may be related to limits of precision of the detection methods themselves. The absorbances and glucose concentrations noted for soybean meal, alfalfa pellets, and moist dog food represent 1.0, 1.4, and 7.7 mg of dietary starch in the respective test portions. It is notable that the distillers grains, for which the 0.1 g test portion would provide approximately 4 mg of dietary starch, had a HorRat value below 2, possibly suggesting a level of dietary starch at and above which precision is improved.

A viable approach to decreasing RSD values for low starch test samples analyzed with the dietary starch method is to increase the size of the test portion in order to increase the amount of analyte to be detected. The idea of increasing the amount of test sample analyzed in order to improve precision by having a greater amount of analyte to measure has been raised (25). Unlike the dietary fiber analyses that may need to restrict test portion size to assure that the extractant remains in excess, starch assays will primarily be restricted by the need to maintain an excess of enzyme to assure complete hydrolysis of the α-glucan. The approach of allowing a range of test portions but a limit on the amount of starch added to the reaction vessel is used by two current AOAC starch methods: AOAC Method **948.02** for starch in plants (26) specifies a use of 0.1–1.0 g of test portion containing approximately 20 mg of starch, and AOAC Method **979.10** for starch in cereals (27) indicates use of a 0.5 g test portion and then specifies "≤1.0 g containing ≤0.5 g starch". In the present method, a limit of 100 mg of dietary starch in each reaction vessel leaves latitude to increase the size of the test portion to that upper limit. Although 0.1 g test portions may be generally adequate, increasing the amount of substrate within the bounds of the assay for feedstuffs with low starch contents may reduce variability of results. The remaining caveat is that as sample quantity is increased, attention must be paid to increasing amounts of interfering substances also brought into the reaction (e.g., antioxidants if the GOPOD assay is used).

With the exceptions of dry ground corn, dairy feed, poultry feed, and corn silage, s_r and s_R were similar within materials (Table 3). The HorRat values obtained in the present study compared favorably to those obtained with AOAC Method **996.11** (10; Table 3). In the collaborative study for that method, starch analyses performed without dimethyl sulfoxide (DMSO)

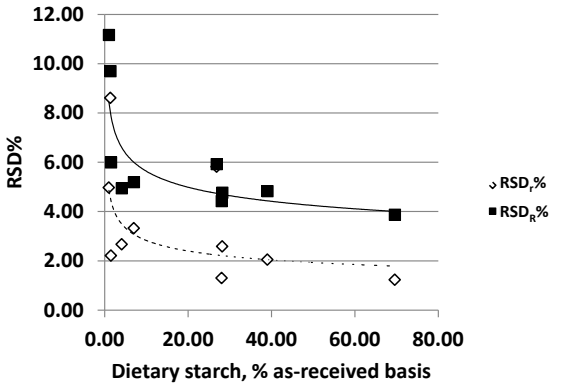

Figure 1. Relationship of dietary starch concentration and RSD values for repeatability within laboratory (RSD_r) and reproducibility between laboratories (RSD_R) obtained in the collaborative study. Equations for the regression lines are RSD_r, % = $4.8616x^{-0.236}$ (R^2 = 0.35; dashed line), and RSD_R,% = $8.4397x^{-0.176}$ (R^2 = 0.66; solid line), where x = dietary starch concentration.

had a starch content of 59.8% as received, and an average HorRat of 2.1 with one value below 2. For the dietary starch collaborative study, the HorRat was less than 2 for six of 10 materials, with an overall average of 2.0 on test materials that averaged 20.7% dietary starch on an as-received basis. Alfalfa pellets and soybean meal had HorRat values of greater than 2.5. As previously discussed, the high RSD_R for these test materials may relate to the combination of their low starch content and the small test portion amount used. Test samples with very low concentrations of the analyte have been reported to give elevated HorRat values (17). The high HorRat value for the dry dog kibble may reflect an issue with homogeneity of the sample, as described previously.

Collaborators' Comments

The collaborators all reported that the assay was not very complicated and was easy to do. They particularly liked additions of all reagents to a single vessel, performing reactions in screw cap tubes, determining total liquid volume as the sum of quantitative volume additions, and making sample solution dilutions by accurate pipetting of volumes. They indicated that they had to work within their laboratories to find tools of acceptable accuracy to make the volume additions, as some of the tools they worked with for other purposes were not adequate. They did report issues with screw cap tube adequacy to hold the needed volume; this was apparently related to differing amounts of glass used by the manufacturers while maintaining the same exterior dimensions of the tubes. That was addressed by describing the screw cap tubes by the volume they needed to contain while allowing adequate room for mixing. With the number of sodium phosphate chemicals available, it was noted that it was crucial to verify and use the exact chemicals specified for the GOPOD reagent. It was also raised that the only extended period to take a break from the assay was during the amyloglucosidase incubation; taking a break after adding water to the fully digested samples resulted in reduced recovery. Development of an approved assay for glucose detection that could be used on a plate reader or automated system was recommended as a way to increase throughput of the assay, which is currently limited by the 30 min period within which samples must be read after incubation in the GOPOD glucose detection assay. Some laboratories had issues with calculating quadratic glucose standard curves; this was resolved by graphing all individual glucose standard solution absorbances data with absorbance on the X-axis and glucose concentration on the Y-axis. Then, a quadratic or second order polynomial regression or "trend" line was graphed through the data. The regression line equation was used for calculation of glucose in test solutions. Collaborators gave extensive input on the method protocol writeup and recommended development of a flow chart for the assay

Recommendations

Based on the results of the collaborative study, the Study Director recommends that the enzymatic-colorimetric method for measurement of dietary starch in animal feeds and pet foods be adopted as Official First Action.

Acknowledgments

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Candidates for 2016 Method of the Year

Sarah Snodgrass and Joshua Arbaugh, West Virginia Department of Agriculture, Charleston, WV

H. Dorota Inerowicz and Keith Binkerd, Office of Indiana State Chemist/Feed Chromatography Laboratory Section, West Lafayette, IN

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AOAC RESEARCH INSTITUTE

Expert Review Panel on Dietary Starches
OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT

ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final version of the Official Chair's Report for the Expert Review Panel on Dietary Starches held on Wednesday, September 10, 2014 during the AOAC Annual Meeting and Exposition at the Boca Raton Resort and Club, 501 East Camino Reel, Boca Raton, Florida 33432.

LARS REIMANN, EUROFINS

Expert Review Panel Chair

Oct 02, 2014

Date

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EXPERT REVIEW PANEL MEETING ATTENDEES

Expert Review Panel Chair (s)
Lars Reimann, Eurofins

Expert Review Panel Members
Sean Austin, Nestle Research Centre
Sneh Bhandari, Silliker, Inc.
Kommer Brunt, Rotating Disc BV
Jon DeVries, Medallion Laboratories
Kai Liu, Eurofins
Barry McCleary, Megazyme International Ireland
Tom Phillips, MD Department of Agriculture
John Szpylka, Silliker, Inc. (Alternate)

Method Authors
Mary Beth Hall, U. S. Department of Agriculture

AOAC Staff
Deborah McKenzie
La'Kia Phillips

Observers
Jason Kong, Ohio Department of Agriculture
Maria Nelson, AOAC Technical Consultant
Ioannis Vrasidas, Eurofins Analytico

EXPERT REVIEW PANEL, METHOD BACKGROUND, AND CONCLUSIONS

Criteria for Vetting Methods to be considered:
AOAC convened the Official Methods of Analysis℠ (OMA) Expert Review Panel for Dietary Starches on Wednesday, September 10, 2014 from 8:00am to 10:00am during the AOAC Annual Meeting and Exposition in Boca Raton, Florida from September 7-10, 2014. The purpose of the meeting will be to 1) Review the Collaborative Study Manuscript/ OMAMAN-13: Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method Collaborative Study (Study Director: Mary Beth Hall, U. S. Department of Agriculture – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA) and to 2) discuss First to Final Action requirements and Feedback mechanisms. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, Method Safety Checklist, Collaborative Study Tables, Collaborative Study Figures and Captions, and the Collaborative Study Protocol.

Criteria for Vetting Experts and Selection Process:
The following eight (8) candidates and one (1) alternate member were submitted for consideration by the Official Methods Board to evaluate candidate methods for Dietary Starches methods as per the Expert Review Panel (ERP) Policies and Procedures. The candidates were highly recommended by the Agricultural Materials Community, have participated in various AOAC activities, including but limited to, Method Centric Committees that were formed under the legacy OMA pathway, and were vetted by the Official Methods Board. The experts are Sean Austin, Sneh Bhandari, Kommer Brunt, Jon DeVries, Kai Liu, Barry McCleary, Tom Phillips, John Szpylka, and the Chair, Lars Reimann.

ERP Orientation:
The ERP members have completed the mandatory AOAC Expert Review Panel Orientation Webinar on Wednesday, July 16, 2014.

Expert Review Panel Meeting Quorum
The meeting of the Expert Review Panel was held in person. A quorum is the presence of seven (7) members or 2/3 of the total vetted ERP, whichever is greater. Eight (8) out of the eight (8) voting members were present and therefore met a quorum to conduct the meeting.

Standard Method Performance Requirements (SMPRs): N/A

Conclusion:
The Expert Review Panel reviewed OMAMAN-13: Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method and adopted this method for First Action Official Method status by a unanimous decision with additional revisions as noted in the meeting minutes.

Subsequent ERP Activities:
ERP members have stated that no additional data is requested to move from First to Final Action. User Feedback and supporting documentation in support of the need for quadratic standard curve is expected for this method to move forward to Final Action Official Method status. ERP members will continue to evaluate the method for 2 years.

MEETING MINUTES

I. Welcome and Introductions

The Expert Review Panel Chair, Lars Reimann welcomed Expert Review Panel members and initiated introductions. The Chair discussed with the panel the goal of the meeting.

II. Review of AOAC Volunteer Policies

Deborah McKenzie presented an overview of AOAC Volunteer Policies, Volunteer Acceptance Agreement and and Expert Review Panel Policies and Procedures which included Volunteer Conflicts of Interest, Policy on the Use of the Association, Name, Initials, Identifying Insignia, Letterhead, and Business Cards, Antitrust Policy Statement and Guidelines, and the Volunteer Acceptance Form (VAF). All members of the ERP were required to submit and sign the Volunteer Acceptance Form. The Expert Review panel openly discussed any potential conflicts of interest. The group approved all of the members after disclaimers were noted.

III. Expert Review Panel Process Overview and Guidelines

Deborah McKenzie presented an overview of the Expert Review panel process. The presentation included information regarding method submission, recruitment of ERP members, composition and vetting expertise, method assignments, meeting logistics, consensus, First Action to Final Action requirements, method modifications, publications, and documentation.

IV. Review of Methods

All members of the ERP presented a review and discussed the proposed collaborative study manuscript for Determination of Dietary Starch in Animal Feeds and Pet Food by an Enzymatic-Colorimetric Method. The method author, Mary Beth Hall of the U. S. Department of Agriculture, was not present to address the concerns of the ERP members. A summary of comments was provided to the ERP members.[1]

MOTION:

Motion by DeVries; Second by Szpylka to adopt this method for First Action Official Methods Status with the requested revisions.

OMA METHOD: Line 376: Include “free from catalase activity”.

EDITORIAL: Line 351: Include “the enzymes should be of a purity meeting the specifications listed in OMA methods 985.29 and 991.43
Line 366: The “amylase” should be listed as “amyloglucosidase”
Line 344: Include activity definitions and assay procedures.
Line 118-122: Please clarify section.

The Expert Review Panel would like to know if GOPOD blank is used as instrument blank will the intercept disappear and negate the need for a quadratic standard curve?

Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous). *Motion Passed.*

[1] Attachment 1: Summary of Expert Reviewer Comments for OMAMAN-13

V. Discuss Final Action Requirements for First Action Official Methods (if applicable)

MOTION:
Motion by DeVries, Second by Liu that no additional data is requested to move from First to Final Action. User Feedback and supporting documentation in support of the need for quadratic standard curve is expected.
Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous). ***Motion Passed.***

VI. Adjournment

	Summary of Method
ER 1	Acceptable
ER 2	It consists of incubation of an aliquot of the sample with thermostable alpha-amylase in pH 5.0 acetate buffer for 1 hr at 100°C with periodic mixing to gelatinize and partially hydrolyze alpha-glucan. Amyloglucosidase is added and mixture is incubated at 50°C for 2 h and mixed. After subsequent addition of water, mixing, clarification, and dilution as needed, free + ezymetically released glucose are measured using a colorimetric glucose oxidase-peroxidase method. Values from a separate determination of free glucose are subtracted to give values of enzymatically-released glucose. Dietary starch = Enzymatically- released glucose multiplied by (162/180) or 0.9 and divided by the as received sample weight (g) used in the assay.
ER 3	Dietary starch is digested to glucose and the increase in glucose level is used to calculate %dietary starch. Potential interferences are either accounted for (inherent glucose) or excluded (deter inherent sucrose digestion and deter maltulose formation).
ER 4	Starch is digested by traditional amylase/amyloglucosidase using gelatinization conditions. Glucose released is measured colorimetrically with adjustment for free glucose in the sample.
ER 5	Ground or homogenized samples are digested with α-amylase and amyloglucosidate in acetate buffer to release glucose from dietary starch. The digestate, after optional dilution, is analyzed for its glucose content. A second sample portion is also assessed for free glucose by treatment with all reagents but the enzymes. The difference of the two glucose result is used to calculate dietary starch content in the sample.
ER 6	Sample (containing up to 100mg of starch) is weighed in duplicate. sodium acetate buffer (pH 5.0) is added to both tubes. Then to one tube alpha-amylase and amylglucosidase are added to hydrolyse the starch. To the other tube no enzymes are added. Samples are then clarified (centrifugation or filtration) and diluted. Aliquots from each tube are then taken for analysis of glucose using the glucose oxidase peroxidase (GOPOD) method, or other suitable validated method for glucose determination. Glucose determined in the untreated sample is subtracted from the glucose determined in the enzyme-treated sample. The result is then multiplied by 0.9 to correct for water uptake during hydrolysis to calculate starch content.
ER 7	good
ER 8	A well performed study. However, the advantages over AOAC Method 996.11 need to be more clearly identified. A significant contribution is the application to samples more relevant to the particular study, but some of the stated general advantages are not substantiated.

	Method Scope/Applicability
ER 1	Animal Feeds and pet foods. 1%-70% starch
ER 2	Animal feedstuffs and pet foods. Limitation in application: The method underestimates dietary starch in feeds and foods whose antioxidant content is known to exceed 10-20 micromol of hydrophilic antioxidant (as ascorbic acid) per 0.1 g of test dry matter. The method in the current format may not be easily applicable to foods/feeds high in phenolic compounds (e.g. beets, red sorghum grain).
ER 3	A wide range of animal and pet feeds were covered in the study. Dry and wet products were included along with a variety of grains as the base material.

ER 4	See method scope and applicability statement.
ER 5	Applicable to pet foods (wet and dry), animal feed, forage, as well as grains.
ER 6	method has been applied to a range of different animal feeds; canned dog food, low starch horse feed, ground corn, complete dairy feed, soybean meal, distillers grains, poultry feed, corn silage, dry dog kibble, alfaalfa pellets. It is applicable for the analysis of "dietary starch" as defined in the introduction of the paper.
ER 7	good
ER 8	Non-resistant starch in animal feeds

	General Comments
ER 1	Positive feedback from collaborators
ER 2	The manuscript describes a method and SLV and its performance in multilaboratory study for dietary Starch (glycogen, maltooligosaccharides, and other alpha-1,6-linked glucose carbohydrates, exclusive of resistant starch). This method is replacement of invalidated AOAC 920.14 due to unavailability of one of the enzyme required in the assay. The described method is more efficient than other methods considered.
ER 3	Measurement of carbohydrates by enzyme-digestion and analysis of the liberated mono-saccharides is an established approach which has worked well for a range of carbohydrates. The collaborative data from this study demonstrates this approach works well for dietary starches due to properly accounting for sucrose & inherent glucose interferences, and in deterring formation of maltulose.
ER 4	Excellent approach
ER 5	This method is similar to older, but now obsolete methods in principal, with better description in choice of enzymes and analysis approach of the glucose contents. This method also simplifies experimental procedures by adding reagents into the same tube until the final dilution step.
ER 6	The principles of the method are good. Enzymes are used to specifically hydrolyse the relevant alpha-glucans in feeds (i.e. starch, maltooligosaccharides, etc) composed of alpha-1,4 and alpha-1,6 linked glucose. Other poly- or oligosaccharides should not be hydrolysed. Resulting glucose is determined using a well established procedure (GOPOD) and free glucose which would interfere is accounted for by running a sample without enzymatic hydrolysis. I don't know if the concept of resistant starch is used in the animal feed world. If yes, it would be good to clarify if the methodology is expected to account for all the starch or only the available starch.
ER 7	none
ER 8	Page 2, line 25. In reference to AOAC Method 996.11, the author refers to the method being “quasi-empirical” and justifies this by stating that “glucose is the analyte detected, but its release is determined by run conditions and specification of enzymes.” The term “quasi-empirical” is unacceptable. This method was run through a full AOAC International interlaboratory evaluation involving 31 laboratories and over this number, the RSDr and RSDR values were similar to those reported in this paper. In reference to the comments about the run conditions and specification of enzymes, of course the method was defined. This is a requirement of any method. It is especially important to specify details of enzymes and particularly purity. This is the reason why so many enzyme based methods have failed in the past. It is dangerous to recommend industrial, or in fact any, enzymes that have not been analysed for activity and purity (contamination with other

	interfering enzymes or sugars etc). AOAC Method 996.11 has also been adapted to run at pH 5. This was evaluated after I had discussions with Mary Beth Hall in 2007 (or 2008). The method works fine at pH 5 and both enzymes are active and stable at this pH. The change to do both incubations at pH 5 is convenient. However, in our hands, the same analytical values were obtained for a number of starch containing samples when sam both incubations were run at pH 5 as compared to running the alpha-amylase incubation at pH 7 (as per 996.11). It is known that a small amount of maltulose can be formed on hydrolysis of starch by alpha-amylase at pH 7 or above. However, this occurs in the industrial hydrolysis of starch which is performed at a starch concentration of approx. 30% w/v. Starch analyses are performed at a starch concentration of just 0.03% (1,000-fold lower concentration). Page 4, line 79. “the use of mildly…excludes the use of alkali or DMSO and thus excludes resistant starch from inclusion in the dietary starch fraction”. This is exactly what is measured in AOAC Method 996.11, unless there is a requirement to also measure RS. So where is the difference? Also, how can the author be sure that RS is not hydrolysed in the gut of horses or chickens (pigs will be much the same as humans). Page 4, line72. Dietary starch is defined and includes glycogen. Of course these methods also measure glycogen and maltodextrins, but glycogen is unlikely to be in an animal ration, and maltodextrins would be rare (perhaps some in distillers grains). Page 6, lines 119-121. Pure corn starch gave a recovery of 99.3%, but in the interlab results, this averaged at just 89,4%. Why? Page 7, point (6). In our laboratory, we have not experienced non-linear color formation with GOPOD reagent over t he range 0 – 1.2 absorbance units. Is this a problem with enzyme purity? Page 8, point (8). Ease of use/efficiency. The advantages claimed are exactly the same advantages as described in AOAC Method 996.11. Where is the difference? Page 9, lines 182-184. Method uses the same temperature for AMFG and glucose analysis. This is already done in 996.11. Page 9. Lines 195-197. Enzyme purity. Enzymes must be free of glucose, but it is essential that they are also free of other enzymes active on other glucose containing polymers e.g. beta-glucan. Industrial AMG preparations are highly contaminated with beta-glucanase and to a lesser extent beta-glucosidase. This requirement should be highlighted. Page 10, lines 214-215. For the participants in the interlab, did you state purity requirements for glucose oxidase and peroxidase. It is essential that high purity enzymes are used. Glucose oxidase is commonly contaminated with catalase and this results in instability and fading of the color formed in this reaction.

	Page 16. Point (b) is missing. Page 16 – Purity and source of alpha-amylase and AMG. Detailed specifications on the source of alpha-amylase are given. However, it must be remembered that these enzymes are made for industrial use. There may be variation from batch to batch in contaminants important in an analytical procedure but of no consequence in the intended industrial application. Industrial AMG cannot be used in analytical procedures because it contains glucose, but more importantly, because they contain contaminating activities that interfere with starch determination in plant samples. As far as I am aware, the Megazyme purified AMG (E-AMGDF) is the only AMG pure enough to use in such assays other than pure AMG, which is too costly to use in such assays. Page 17, line 336. Change “amylase” to “amyloglucosidase” Page 17 (e). A statement should be made about the required purity of glucose oxidase and peroxidase. Page 17, line 378. Phenol is generally not used in glucose determination reagents because it is carcinogenic and also is not very stable. The chemical most commonly used in its place is *p*-hydroxybenzoic acid.
ER 9	For me the term "dietary starch" is new, especailly in connection with animal feed and pet food. Fromenergetic viewpoint, I can agree to include maltodextrins, glycogen fromanimal and microbial origin in the new term dietary starch. However I have problems what to do with the4 different types of resistant starhes, the RS1, RS2, RS3 and RS4. Starch incubation with alpha-amylase at 100 C will hydrolyse the RS1, RS2 and RS4 resistant starch but certainly not the RS3 resistant starch, the so-called retrograded starch. Different animals have different intestinal tracks, for example, pets, pigs, cows some can digest resistant starches, others not. So the content of dietary starch in a feed sample depends also on which kind of animal consumes the feed.The for digestion available "dietary starch" in one sample containing resistant starch categories RS1+RS2+RS3+RS4 is most likely different for pets (originally carnivores and less capable to digest native starches), pigs, cows.

	Method Clarity
ER 1	Positive feedback from collaborators
ER 2	Good with the exception how the limitation of the method in application to matrices containing hydrophilic antioxidant contents/activity exceeding 10-20 micromol as ascorbic acid) per 0.1 g of test dry matter.
ER 3	Easy to read. No issues.
ER 4	Well written and understandable

ER 5	Satisfactory
ER 6	Method is clearly written I didn't have problems following it, with the exception of the units used for the enzyme activities. It would be preferable for the authors to define the units of activity for each enzyme since definitions vary from manufacturer to manufacturer. This will be fundamental if the enzymes used need to be replaced with others.
ER 7	good
ER 8	Well thought through study and well written

	Pros/Strengths
ER 1	Single vessel
ER 2	Relatively more efficient method. Very well studied and validated in SLV. 15 labs. collaboratively studied the method and analyzed 10 homogenous test materials (animal feeds and pet foods) using the described method for dietary starch (ranging starch contents of 1-70%). The average within lab. Repeatability as sr for % Dietary starch was 0.49 with a range of 0.03 to 1.56, and among –laboratory repeatability of standard deviation sR averaged 0.96 with a range of 0.09 to 2.69. HORRAT averaged 2.0 for all test samples and 1.9 for samples containing dietary starch more than 2%.
ER 3	Measurement of carbohydrates by enzyme-digestion and analysis of the liberated mono-saccharides is an established approach which has worked well for a range of carbohydrates. The collaborative data from this study demonstrates this approach works well for dietary starches due to properly accounting for sucrose & inherent glucose interferences, and in deterring formation of maltulose. Dietary starch is digested to glucose and the increase in glucose level is used to calculate %dietary starch. Potential interferences are either accounted for (inherent glucose) or excluded (deter inherent sucrose digestion and deter maltulose formation).
ER 4	Traditional chemistry that has been well studied. Can be carried out in modestly equipped laboratories by technical personnel with modest training.
ER 5	Relatively straightforward procedures Satisfactory recovery on glucose and corn starch. Low interference from sucrose , β-glucan and cellulose. Good repeatability and reproducibility.
ER 6	- A simple method that does not need specialized equipment. - option to use alternative methods for glucose analysis is mentioned if a lab does not wish to use the GOPOD assay
ER 7	no comment
ER 8	The specific advantages of this method over AOAC Method 996.11 are not clear. With both methods, good reproducibility and recovery of starch was obtained over a wide range of samples. This method is no easier to perform than 996.11.

	Cons/Weaknesses
ER 1	None
ER 2	The method underestimates dietary starch in feeds and foods whose antioxidant content is known to exceed 10-20 micromol of hydrophilic antioxidant (as ascorbic acid) per 0.1 g of test dry matter. The method in the current format may not be easily applicable to foods/feeds high in phenolic compounds (e.g. beets, red sorghum grain).

ER 3	Spectrophotometric measurement does work well and is easy, quick, and reliable. Quantitative of sugars by HPLC is also simple (a bit more expensive though) but will allow tracking of sucrose to assure its digestion did not occur. The method's steps do prevent sucrose digestion by relying on high-purity enzymes. Since these enzymes are more expensive, laboratories using lower cost enzymes would be at risk of reporting less accurate, higher values.
ER 4	Lack of sophisticated instrumentation will be unappealing to those inclined to high level tech methods.
ER 5	Quadratic fit may be difficult for some users to use, and automation of the whole quantitation process is somewhat difficult to achieve. Scaling up is somewhat limited because of the need to measure absorbency within 30 min of GOPOD reaction. High content of anti-oxidant will prevent accurate determination of glucose, forcing other glucose detection methods into consideration.
ER 6	- potential interference of substances with anti-oxidant activity (if this is unknown it needs to be assessed somehow, or an alternative glucose assay should be used) - although it is mentioned that glucose assays other than GOPOD can be used, it does not appear to have been tested or validated. - it is mentioned that leaving the sample (taking a break) after dilution of fully digested samples has an impact on recovery - but why should that be the case?
ER 7	none
ER 8	This is a good method, but would appear not to be an improvement over AOAC

	Supporting Data Comments
ER 1	Impressive data package
ER 2	15 labs. collaboratively studied the method and analyzed 10 homogenous test materials (animal feeds and pet foods) using the described method for dietary starch (ranging starch contents of 1-70%). The average within lab. Repeatability as sr for % Dietary starch was 0.49 with a range of 0.03 to 1.56, and among –laboratory repeatability of standard deviation sR averaged 0.96 with a range of 0.09 to 2.69. HORRAT averaged 2.0 for all test samples and 1.9 for samples containing dietary starch more than 2%.
ER 3	Excellent study
ER 4	Excellent data package. Well done study.
ER 5	Well-organized summary tables about statistics of all matrix results Good study on the glucose standard responses across different batches
ER 6	This looks to be a straight forward assay which did not appear to be problematic for most of the labs involved in the MLT. The authors have mentioned that alternative assays for glucose could be used instead of GOPOD (and may be essential for samples with high anti-oxidant contents). It would be interesting to know if this has been tested in any of the labs because although it is mentioned it does not appear to have been verified.
ER 7	good
ER 8	Method should be accepted with some changes to text

	Method Optimization
ER 1	Done
ER 2	The method has been optimized for its efficiency and better recovery of starch.
ER 3	Keep as written (see comment in Cons/Weaknesses for optional digestion)
ER 4	No further work needed.

ER 5	Same temperature for both enzymatic procedures, allowing better efficiency. Changed to quadratic curve due to the slight non-linearity of the standards.
ER 6	It would be interesting to understand why leaving the sample (taking a break) after dilution of fully digested samples has an impact on recovery (line 699, p31)
ER 7	good
ER 8	n/a

	Analytical Range
ER 1	1-100%
ER 2	0-100 mg starch in the assay
ER 3	Range studied was 1.00% - 69.6%. Corn starch was used as a spiking agent which suggests this material can be tested directly on this material (89% dietary starch) as long as enzymes are keep in sufficient excess/
ER 4	See method collaborative study report.
ER 5	~1% to 100%
ER 6	about 1 (lowest amount in samples tested in MLT) - 100% starch (considering corn starch used as control)
ER 7	good
ER 8	Acceptable

	LOQ
ER 1	Approx. 0.3% (probably a little larger)- definitely less than 1%
ER 2	0.9% of starch sample weight basis
ER 3	0.3%. Acceptable limit.
ER 4	See method collaborative study report.
ER 5	0.3%
ER 6	This has been estimated as 0.2% dietary starch by using reagent blanks. The approach seems reasonable, although one may expect the practical LoQ to be higher when applied to samples (and is probably not independent of the free glucose content of a sample)
ER 7	good
ER 8	Acceptable

	Accuracy/Recovery
ER 1	99.3 pure corn starch, 90@ control corn starch.
ER 2	89.9% +/- 3.7%
ER 3	993.8% wi+/- 0.8% is excellent
ER 4	See method collaborative study report.
ER 5	Pure corn starch: 99.3% ± 0.8% (Theoretical = 100%) Corn Starch: 89.9% ± 3.7% (Estimated = 89.4)
ER 6	This does not appear to have been extensively tested. Pure starch products have been assayed and the recoveries are greater than 95%, Dextrins appear to be more problematic, but this does not seem to have been discussed.
ER 7	good

ER 8	Good

	Precision
ER 1	Average RSDr 3.5%
ER 2	RSDr % = 1.23 - 8.61%
ER 3	Acceptable. Soybean meal was the highest but this was likely due to a possible lower degree of sample homogeneity.
ER 4	See method collaborative study report.
ER 5	2-3% most samples; >8% Alfalfa pellets (low level @ ~1%); ~6% Dry Dog Kibble; 5% Soybean Meal (low level @~1%)
ER 6	RSD(r) varies from 1.2 - 8.6 %, and is generally below 5% which I would generally regard as acceptable.
ER 7	good
ER 8	Good

	Reproducibility
ER 1	Average RSDR 6.1%
ER 2	RSDR% = 3.87 - 11.16%
ER 3	Acceptable. Soybean meal was the highest but this was likely due to a possible lower degree of sample homogeneity.
ER 4	See method collaborative study report.
ER 5	4-6% most samples; ~10% for Alfalfa pellets and Soybean Meal
ER 6	RSD(R) varies from 3.9- 11.2 %, and is generally below 6% which I would also consider acceptable.
ER 7	good
ER 8	Good

	System Suitability
ER 1	Good systems suggested (Starch, sucrose, glucose)
ER 2	The use of corn starch as control sample to evaluative quantitative recovery in the assay.
ER 3	see above
ER 4	Definitely suitable for purpose
ER 5	N/A
ER 6	The use of enzymatic hydrolysis to convert starch to glucose, and the GOPOD assay to specifically assay the starch means the method is very selective. Potential interferences have been identified and suitable controls are mentioned.
ER 7	good
ER 8	Acceptable

	First Action Recommendation
ER 1	Yes
ER 2	Yes, I do recommend the method to be adopted as First Action Method by ERP after authors have explained the following two limitation of the method in application. 1. The method in the current format is not be easily applicable to foods/feeds high in phenolic compounds (e.g. beets, red sorghum grain). 2. Oats beta-glucan interfere in the assay and provide values above LOD = 0.31 +/- 0.09%.
ER 3	Yes. Recommend consideration of allowing HPLC as an option to measure liberated glucose to calculate %dietary starch. This approach would also measure free, inherent glucose and track if sucrose-digestion has occurred.
ER 4	Yes
ER 5	Yes.
ER 6	Yes
ER 7	yes
ER 8	Yes

	After First Action Recommendation
ER 1	Use feedback
ER 2	NA
ER 3	Recommend consideration of allowing HPLC as an option to measure liberated glucose to calculate %dietary starch. This approach would also measure free, inherent glucose and track if sucrose-digestion has occurred. Decision needed if single or multiple lab work is needed to verify.
ER 4	Just the normal 2 year feedback period. Collaborative is completed and complete.
ER 5	N/A
ER 6	It would be good to test the performance of the method when an alternative glucose assay is used. Clarify the reason why dextrin recovery is low.
ER 7	no
ER 8	That included in the text above. (Please clarify)

AOAC Official Method 2014.03
Gluten in Rice Flour and Rice-Based Food Products
G12 Sandwich ELISA
First Action 2014

(Applicable for determination of gluten in rice flour and rice-based unprocessed and processed foods as evaluated in a multilaboratory study.)

Caution: Wear protective gloves and safety glasses. The stop solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water (*see* Material Safety Data Sheet). The extraction solution contains chemicals which are harmful to health. Perform sample extraction under a chemical hood and avoid contact with skin. Dispose of all materials, containers, and devices appropriately after use.

See Table **2014.03A** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

The method is based on an enzyme immunoassay format using a monoclonal G12 antibody that can determine gluten derived from wheat, rye, barley, and cross-bred varieties. The G12 antibody binds to the celiac toxic amino acid sequence QPQLPY and related sequences in rye and barley. The antibody detects prolamins in nonheated and heated food by using a specific proprietary extraction solution. No cross-reactivity has been determined to maize, rice, teff, millet, buckwheat, quinoa, amaranth, and soy (*see* Table **2014.03B**).

Gluten is extracted from samples using proprietary extraction solution containing reducing agents followed by ethanol extraction. After centrifugation the supernatant is used in a sandwich enzyme-linked immunoassay. When incubated on monoclonal antibody-coated microwells, the analyte is forming an antibody-antigen complex. After a washing step, an enzyme-conjugated monoclonal antibody is applied to the well and incubated. After a second washing step, an enzyme substrate is added and blue color develops. The intensity of the color is directly proportional to the concentration of gluten in the sample or standard. A stop solution is then added which changes the color from blue to yellow. The microwells are measured optically using a microwell reader with a primary absorbance filter of 450 nm (OD450). The optical densities of the samples are compared to the standards and an interpolated result is determined.

B. Apparatus

The apparatus specified has been tested. Equivalent apparatus may be used.

(**a**) *Osterizer blender*.—Used for homogenization of sample (Sunbeam-Oster, Ft. Lauderdale, FL, USA).

(**b**) *Centrifuge tubes*.—50 mL for extraction (Star Labs International GmbH, Hamburg, Germany).

(**c**) *Glassware*.—Wash bottle (1000 mL) and graduated cylinders.

(**d**) *Water bath*.—Grant Sub Aqua 12 (Grant Instruments, Cambridgeshire, UK).

(**e**) *Stuart roller mixer*.—Bibby Scientific Ltd (Staffordshire, UK).

(**f**) *Bench top centrifuge*.—Sigma 1-14 (Sigma Laborzentrifugen, Osterode am Harz, Germany).

(**g**) *Centrifuge tubes*.—2 mL; for sample dilution (Star Labs International GmbH).

(**h**) *Micropipet*.—Accurately delivering 100 µL ± 1%.

(**i**) *Microtiter plate reader with a 450 nm filter*.—Thermo Fisher Scientific (Shanghai, China).

C. Reagents

Items (**a**)–(**i**) are available as a test kit (AgraQuant Gluten G12 ELISA®, Romer Labs UK Ltd, Runcorn, UK). All reagents are stable for 12 months from date of manufacture at 2–8°C (36–46°F). Refer to kit label for current expiration.

(**a**) *Antibody-coated microwell strips*.—Monoclonal antibodies are coated in 20 mM phosphate buffered saline (PBS) onto a set of 12 eight-microwell strips (NUNC, Roskilde, Denmark).

(**b**) *Gluten ready-to-use standards (antigen)*.—Five vials containing 1.2 mL of each gluten G12 standard (0, 4, 20, 80, and 200 mg/kg labeled as ppm), prepared by vital wheat gluten dissolved in 60% ethanol at a concentration of 1 mg/mL. Solution is further diluted in 20 mM PBS–Tween (0.9% sodium chloride, 0.07% Tween 80) containing 0.25% fish gelatin (Sigma) to 0, 10,

Table 2014.03A. Performance statistics for overall G12 sandwich ELISA results

Parameter	Symbol	Sample ID[a]											
		1	2	3	4	5	6	7	8	9	10	11	12
Total No. laboratories	*P*	17	18	18	18	16	18	18	16	17	18	18	18
Total No. replicates	Sum [n(L)]	34	36	36	36	32	36	36	32	34	36	36	36
Overall mean of all data (grand mean; mg/kg)	xbarbar	1.6	13.5	26.2	101.2	0.1	6.2	13.1	63.5	4.1	14.9	26.6	112.7
Repeatability SD, mg/kg	s_r	0.8	2.5	8.1	14.8	1.2	1.2	1.3	5.1	1.9	1.5	4.3	20.4
Reproducibility SD, mg/kg	s_R	1.9	4.0	11.6	31.8	1.2	1.8	2.5	13.5	2.8	4.5	8.9	33.2
Repeatability RSD, %	RSD_r	48.2	18.5	30.7	14.7	2348	19.2	10.2	8.0	46.2	10.4	16.2	18.1
Reproducibility RSD, %	RSD_R	115.8	29.6	44.2	31.4	2348	28.3	19.1	21.2	69.0	30.3	33.6	29.4
Bias (mg/kg) observed-nominal		1.6	3.5	6.2	1.2	0.1	–3.8	–6.9	–36.5	–0.4	–0.1	2.6	10.7
Recovery, % = observed/nominal × 100			135.0	131.0	101.2		62.0	65.5	63.5	91.1	99.3	110.8	110.5

[a] 1 = Gluten-free rice flour; 2 = rice flour 10 mg gluten/kg; 3 = rice flour 20 mg gluten/kg; 4 = rice flour 100 mg gluten/kg; 5 = gluten-free chocolate cake; 6 = chocolate cake 10 mg gluten/kg; 7 = chocolate cake 20 mg gluten/kg; 8 = chocolate cake 100 mg gluten/kg; 9 = crisp bread 4.5 mg gluten/kg; 10 = crisp bread 15 mg gluten/kg; 11 = crisp bread 24 mg gluten/kg; and 12 = crisp bread 102 mg gluten/kg.

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Table 2014.03B. Cross-reactivity of the G12 antibody (G12 antibody shows no cross-reactivity to various nuts, oils, seeds, starches, or gluten-free grains)

Food category	Food sample	Romer extraction solution, mg/kg gluten	Gluten, %
Gluten-containing grains	Wheat flour	72222	7.2
	Barley (Cumion)	292390	29.2
	Durum wheat	15733	1.6
	Spelt (Ostro)	81926	8.2
	Rye (Capitan)	41577	4.2
Naturally gluten-free grains	Soya bean	<4	
	Soya mince	<4	
	Buckwheat	<4	
	Rice flour	<4	
	Quinoa	<4	
	Corn kernels	<4	
	Teff flour	<4	
	Millet	<4	
Oats	Bastion	4.3	
	00-61 Cn	7.4	
	Brachan	<4	
	Husky	6.3	
	Fusion	6.6	
Nuts	Pecan	<4	
	Walnut	<4	
	Almond	<4	
	Cashew	<4	
	Macadamia	<4	
	Peanut	<4	
	Hazelnut	<4	
	Pine nut	<4	
	Pistachio	<4	
Seeds	Golden linseed	<4	
	Brown linseed	<4	
	Poppy	<4	
	Sesame	<4	
	Mustard	<4	
Oils	Hazelnut oil	<4	
	Walnut oil	<4	
	Vegetable oil	<4	
	Sunflower oil	<4	
Starches	Tapioca starch	<4	
	Wheat starch	<4	
	Potato starch	<4	
Miscellaneous	Amaranth	<4	

50, 200 and 500 ng/mL gluten, calibrated to the WGPAT gliadin (86% highly purified gliadin from 40 different European wheat varieties).

(**c**) *Conjugate solution (peroxidase-labeled antibody, ready-to-use)*.—One bottle containing 13 mL.

(**d**) *Substrate solution (stabilized peroxide substrate and 3,3′,5,5′-tetramethyl-benzidine in a dilute buffer solution)*.—One bottle containing 15 mL.

(**e**) *Stop solution (1 N H_2SO_4)*.—One bottle containing 15 mL.

(**f**) *Diluent buffer*.—One bottle containing 20 mL of 5× concentrated diluent buffer. Contains a final concentration of 20 mM PBS-Tween (0.9% sodium chloride, 0.07% Tween 80) with 0.25% fish gelatin (Sigma) and 0.01% Proclin as a preservative.

(**g**) *Wash buffer*.—One bottle containing 60 mL of 10× concentrated wash buffer. Contains a final concentration of 20 mM PBS-Tween (0.9% sodium chloride, 0.05% Tween 20) with 0.01% Proclin as preservative.

(**h**) *Extraction solution*.—One bottle containing 105 mL of ready-to-use proprietary extraction solution containing reducing agents.

(**i**) *Fish gelatin*.—One sachet containing 10 g.

Additional reagents needed, but not provided with the test kit:

(**j**) *Distilled or deionized water*.

(**k**) *Ethanol*.—80% (v/v).

D. General Instructions

Due to the sensitivity of the assay, a gluten-free environment must be maintained. It is preferable to perform the assay in a separate room from that used for sample preparation and extraction. Make sure balance and the surrounding space, as well as equipment such as spatulas, are clean. Cleaning can be done by using a 70% alcoholic solution. Spatula should be cleaned after each sample weighing by a 70% alcoholic solution.

Store kit at 2–8°C (35–46°F) and let all components equilibrate to 20–25°C (68–77°F) before use.

Include ready-to-use standards in duplicates to each run of samples. Use separate pipet tips for each standard and each sample extract to avoid cross-contamination.

It is recommended that an eight-channel pipettor is used to perform the assay. No more than 48 samples and standards total should be run in one experiment when using an eight-channel pipettor (24 when samples and standards are added in duplicate, e.g., six test strips). If using only single-channel pipets, it is recommended that no more than a total of 16 samples and standards are analyzed in one experiment (eight when standards and samples are added in duplicate, e.g., two test strips).

E. Preparation of Components Delivered with the Kit

(**a**) *Sample dilution buffer*.—Dilute diluent buffer concentrate 1:5 with distilled water (e.g., add 20 mL of concentrated diluent buffer to 80 mL distilled water). Dilution buffer may be used within 24 h, if stored at 4°C.

(**b**) *Wash buffer*.—If a precipitate is formed during storage of the wash buffer concentrate, the concentrate should be warmed up until it is dissolved. Dilute wash buffer concentrate 1:10 with distilled water (e.g., add 10 mL of concentrated wash buffer to 90 mL distilled water). Wash buffer may be used within 1 week, if stored at 4°C.

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F. Sample and Test Portion Preparation

Obtain a representative sample and homogenize a minimum of 5 g in a mortar or blender as fine as possible. Weigh out 0.25 g of homogenized sample into a vial with a minimum 10 mL capacity, which can be tightly sealed. For chocolate-containing samples, additionally add 0.25 g of powdered fish gelatin. Add 2.5 mL extraction solution (under a fume/chemical hood), close vials, and mix vigorously on a vortex. Visually check for clumps, and continue mixing until samples are well dispersed in the extraction solution.

Incubate at 50°C (122°F) for 40 min in a water bath. Allow the extracts to cool to room temperature and add 7.5 mL of 80% ethanol; mix well. Shake for a total of 60 min at room temperature (20–25°C/68–77°F) with a rotary shaker. (After about 30 min in the rotator, check the vials visually if all sample material has suspended in the liquid. If clumps have formed, vortex and let the vials rotate for the second 30 min to complete the extraction procedure).

Centrifuge samples for 10 min at 2000 × *g* to obtain a clear aqueous layer between the particulate sediment and supernatant. Note, in some cases, a thin fatty layer creaming on top of the supernatant. Collect the aqueous supernatant (extract) and transfer into a new vial. Dilute supernatant at least 1:10 (0.1 + 0.9 mL) with prediluted sample dilution buffer (depending on the expected prolamin content of the sample). If prediluted samples are not immediately used for determination by ELISA, close vials and keep in the dark at room temperature (20–25°C/68–77°F) for a maximum of 7 days until ELISA experiments.

G. Determination (Assay)

Bring all reagents to room temperature (20–25°C, 68–77°F) before use.

Use dilution of the sample extract to carry out ELISA experiments. Run standards and diluted sample extracts in duplicate. Place an appropriate number of antibody-coated microwells in a microwell strip holder. Record standard and sample positions.

Using a single-channel pipettor, add 100 µL of each ready-to-use standard or prepared sample into the appropriate well. Use a fresh pipet tip for each standard or sample. Make sure the pipet tip has been completely emptied.

Incubate at room temperature (20–25°C, 68–77°F) for 20 min. Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with diluted wash buffer, and then emptying the buffer from the microwell strips. Repeat this step four times for a total of five washes. Take care not to dislodge the strips from the holder during the wash procedure. Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel all of the residual buffer after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

Measure the required amount of conjugate from the green-capped bottle (about 120 µL/well or 1 mL/strip) and place in a separate container (e.g., reagent boat when using the eight-channel pipettor). Using an eight-channel pipet, dispense 100 µL of conjugate into each well.

Incubate at room temperature (20–25°C, 68–77°F) for 20 min. Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with diluted wash buffer, and then emptying the buffer from the microwell strips. Repeat this step four times for a total of five washes. Take care not to dislodge the strips from the holder during the wash procedure. Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel all of the residual buffer after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

Measure the required amount of substrate from the blue-capped bottle (about 120 µL/well or 1 mL/strip) and dispense into a separate container (e.g., reagent boat for an eight-channel pipettor).

Pipet 100 µL of the substrate into each microwell using an eight-channel pipettor. Incubate at room temperature (20–25°C, 68–77°F) for 20 min in the dark.

Measure the required amount of stop solution from the red-capped bottle (about 120 µL/well or 1 mL/strip) and dispense into a separate container (e.g., reagent boat for an eight-channel pipet).

Pipet 100 µL of stop solution into each microwell using an eight-channel pipettor. The color should change from blue to yellow.

H. Reading

Eliminate air bubbles prior to reading wells as they are likely to affect analytical results.

Read the absorbance of wells with a microwell reader using a 450 nm filter. Record OD readings for each microwell.

I. Calculations

Use unmodified OD values or OD values expressed as a percentage of the OD of the 200 ppm standard to construct a dose-response curve using the five standards (0, 4, 20, 40, and 200 ppm gluten). Gluten concentration given for the standards already consider sample preparation and 1:10 dilution according to method protocol. Gluten concentrations of samples can be calculated by interpolation from this standard curve using a point-to-point calculation.

If a sample contains gluten levels higher than the highest standard (>200 ppm), the sample extract should be further diluted with dilution buffer such that the diluted sample results are in the range of 4 to 200 ppm and reanalyzed to obtain accurate results. The dilution factor must be included when the final result is calculated.

J. Criteria for Acceptance of Standard Curve

An example for the calibration curve is shown in the Certificate of Analysis included in each test kit. Higher OD values of the absorbance at 450 nm compared to the certificate may indicate insufficient washing or gluten contamination. For samples showing OD values higher than the 200 ppm standard, a further dilution and repeated analysis is recommended. The additional dilution factor must be taken into consideration during calculation.

Any coloration of the substrate solution prior to the analysis or OD value of less than 1.1 absorbance units for 200 ppm standard may indicate instability or deterioration of reagents.

Reference: *J. AOAC Int.* **98**, 103(2015)
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Posted: March 9, 2015

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Candidates for 2016 Method of the Year

FOOD COMPOSITION AND ADDITIVES

Gluten in Rice Flour and Baked Rice Products by G12 Sandwich ELISA: First Action 2014.03

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The Protein and Enzymes Technical Committee of American Association of Cereal Chemists initiated a collaborative study to confirm whether the G12 antibody-based sandwich ELISA test kit is able to detect gluten in the lower mg/kg (ppm) level. Twenty laboratories investigated 24 heat-treated and non-heat-treated blind-coded samples with incurred gluten levels up to 100 mg/kg. The method has been validated for testing foods to conform to the defined Codex thresholds for gluten in gluten-free products at less than 20 mg gluten/kg. The collaborative study showed that low levels of gluten could be detected by G12 Sandwich ELISA with reproducibility RSD_R of 32% and repeatability RSD_r of 16%. Incurred samples showed a recovery between 62 and 135%. It is recommended that the method be accepted by AOAC as Official First Action.

AgraQuant® Gluten G12 is a sandwich ELISA for quantification of gluten from wheat, rye, barley, and cross-bred varieties in various foodstuffs. The G12 antibody utilized in the test kit binds to the celiac toxic amino acid sequence QPQLPY and related sequences in rye and barley (1, 2). A homogenized sample is extracted with ethanol and a proprietary extraction solution containing reducing agents. The gluten determination is based on a microtiter plate coated with specific monoclonal G12 antibody. Gluten is detected with a peroxidase-labeled G12 antibody. The determination can be done in 60 min. Ready-to-use standards of the ELISA test kit are calibrated against the Working Group on Prolamin Analysis and Toxicity (WGPAT) gliadin standard material and cover a range from 4 to 200 mg gluten/kg sample (*see* Figure 1). The preparation of ready-to-use standards was described at Halbmayr-Jech et al. (3).

Single-laboratory validation (SLV), performed by Romer Labs UK Ltd in May 2011, determined an LOD of 2 mg gluten/kg sample and an LOQ of 4 mg gluten/kg sample (*see* Table 1) as well as a recovery rate ranging from 90 to 145% (*see* Table 2) for the Gluten G12 Sandwich ELISA assay. Coefficient of variation for repeatability and lot-to-lot variation (reproducibility) was 15% or less determined within the SLV (*see* Tables 3–5). The AgraQuant Gluten G12 kit furthermore produced results similar to those assigned values for the current Codex type I approved R5 Mendez method in three Food Analysis Performance Assessment Scheme (FAPAS) rounds in 2011 (*see* Table 6).

The Gluten G12 Sandwich ELISA assay has been evaluated in a collaborative study with 20 participants. The main target for an allowable immunogenic gluten method according to the Codex Alimentarius is that it should have a detection limit of 10 mg/kg or below (4). This paper reports the findings of the collaborative study and discusses the results in relation to current thresholds (20 mg/kg) for gluten-free products.

Collaborative Study

Study Design

The study was conducted on 12 different food samples prepared in the laboratory of the Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany. Blind-coded samples in duplicate, ELISA test kits including extraction solution, method instructions, and result reporting sheets were sent to all participating laboratories.

Collaborators

The collaborative study was coordinated by Clyde Don, Foodphysica, Driel, The Netherlands. Twenty laboratories from the food producing industry, universities, governments, contract

Received August 5, 2014.
The method was approved by the Expert Review Panel for Food Allergens-Gluten as First Action.
The Expert Review Panel for Food Allergens-Gluten invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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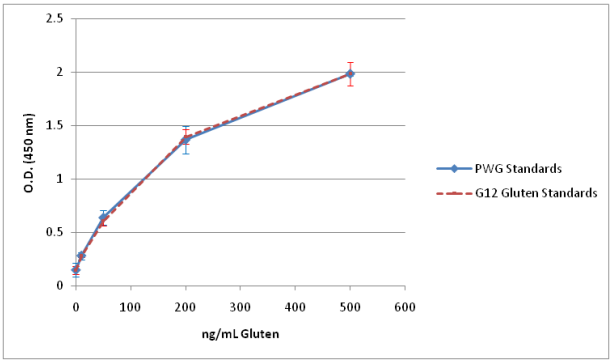

Figure 1. Calibration curve of monoclonal G12 ELISA: Six replicates each of the Vital wheat gluten and PWG gliadin standards were run on the AgraQuant Gluten G12 test kit. Error bars indicate 2 × SD of standard.

laboratories, and kit suppliers from Europe, United States, Canada, Australia, and New Zealand participated in the collaborative study. All collaborators are listed in the *Acknowledgments* section.

Description and Preparation of Samples

The following 12 samples were prepared for the collaborative study: gluten-free rice flour, rice flour containing 10 mg gluten/kg, rice flour containing 20 mg gluten/kg, rice flour containing 100 mg gluten/kg, gluten-free chocolate cake, chocolate cake containing 10 mg gluten/kg, chocolate cake containing 20 mg gluten/kg, chocolate cake containing 100 mg gluten/kg, crisp bread containing 4.5 mg gluten/kg, crisp bread containing 15 mg gluten/kg, crisp bread containing 24 mg gluten/kg, and crisp bread containing 102 mg gluten/kg. Initial target concentrations of the crisp bread samples had been 0, 10, 20, and 100 mg/kg, but a gluten contamination occurred during the preparation of these samples. The contamination was independently confirmed with another antibody-based ELISA, giving further reason to allow a re-estimation of gluten content of respective samples.

All ingredients except wheat flour were confirmed to be free of gluten contamination before use by means of the G12 Sandwich ELISA, which was also used in this collaborative study.

The gliadin content of wheat flour of the German cultivar 'Genius' was determined by an extraction/RP-HPLC method as described by Wieser et al. (5). HPLC absorbance values measured at 210 nm were converted to protein concentration using a standard solution of reference gliadin from the Prolamin Working Group (6). The gliadin content of the wheat flour sample was 67.8±0.16 g/kg (n = 3) on an "as is" basis. The gluten content of the wheat flour was calculated according to Codex (gluten = 2 × prolamin) and was 135.6 g/kg.

Samples were heat-treated to a different extent during processing as found in consumer products. Rice flour was used "as is" (not heat-treated) and represented a base material for the production of gluten-free rice based products. Gluten-free rice flour was provided by General Mills (Minneapolis, MN). Gluten-containing stock rice flour with a gluten concentration of 200 mg/kg was prepared by mixing wheat flour into rice flour and subsequently diluting the mixture with rice flour. Gluten-containing rice flour samples were prepared as follows: 10 mg/kg, 17.5 g stock rice flour was mixed with 332.5 g gluten-free rice flour; 20 mg/kg, 35 g stock rice flour was mixed with 315 g gluten-free rice flour; and 100 mg/kg, 175 g stock rice flour was mixed with 175 g gluten-free rice flour. Mixtures were shaken in an overhead shaker for at least 1 h.

Chocolate cake represented a product that had been moderately heat-treated, but with typical chocolate components that are known to be challenging for ELISA tests. Gluten-free chocolate

Table 1. Calculation of LOD from single-laboratory validation data: 47 replicates of buffer blanks were run over 10 individual AgraQuant Gluten G12 assays. The LOD was determined by calculating the mean OD of the 0 mg/kg standard + 3 SD and then reading this value back off the standard curve. The lower LOQ was determined by the lowest standard of concentration.

Standard, mg/kg	Mean (OD)	SD (OD)	CV, % (OD)	Mean + 3 SD (OD)	LOD, mg/kg
0	0.14	0.03	21.15	0.23	2.00

Table 2. Spike recovery data from single-laboratory validation data: samples were tested both in their original state and spiked with 10 mg/kg of Vital wheat gluten extract. Percentage recovery was calculated against a positive control spiked into extraction buffer. Recovery of 10 mg/kg spike was achieved from a range of processed food samples within an acceptable range (90–145%). The addition of gelatin to the extraction solution significantly increased the extraction efficiency from chocolate

		Romer extraction solution		
Sample	No spike	Spike (10 ppm gluten)	Spike CV, %	Recovery, %
Crisps	<4	12.6	1.35	134.0
Chocolate	<4	<4	NA[a]	NA
Chocolate + gelatin	<4	10.3	1.84	109.6
Cheesy corn snack	<4	8.5	5.33	90.4
Paprika	<4	10.8	0.16	114.9
Chicken	<4	9.7	2.44	103.2
Yogurt	<4	9.4	0.88	100.0
Curry sauce	<4	12.4	0.31	131.9
Margarine	<4	13.6	7.00	144.7
Positive control	NA	9.4	1.31	100.0

[a] NA = Not applicable.

Table 3. Single-laboratory validation data on repeatability using a single kit: 10 replicates of the standard curve were run using a single AgraQuant Gluten G12 test kit. Mean OD values, SD, and CV are shown below. All CV values for intra-assay analysis were less than 15%, meeting the manufacturer's QC criteria

Standard, mg/kg	Mean (OD)	SD (OD)	CV, % (OD)
0	0.138	0.018	12.80
4	0.359	0.035	9.88
20	0.698	0.058	8.34
80	1.340	0.073	5.43
200	1.877	0.109	5.82

Candidates for 2016 Method of the Year

Table 4. Single-laboratory validation data on repeatability using different kits of the same batch: 10 individual AgraQuant Gluten G12 assays containing all the standards were run. Mean OD values, SD, and CV are shown below. All CV values for interassay analysis were less than 15%, meeting the manufacturer's QC criteria

Standard, mg/kg	Mean (OD)	SD (OD)	CV, %
0	0.12	0.01	10.79
4	0.28	0.04	14.26
20	0.67	0.05	7.89
80	1.29	0.13	10.33
200	2.00	0.17	8.70

cake was prepared by mixing one bag (425 g) of gluten-free cake mix (Betty Crocker Gluten-Free Cake Mix, General Mills) with 237 mL water, 112 g baking fat (Sanella, Unilever, Hamburg, Germany), and three eggs with a hand mixer at high speed for 5 min. The mass was poured into a round baking tin [diameter (Ø) = 25 cm] and baked in an oven at 170°C for 45 min. The cake was subjected to cooling for 1 h, sliced with a knife, and air-dried at room temperature (22°C) overnight (16 h). The air-dried cake was then lyophilized and ground with a household grinder (Model 836.820 1, Privileg, Fürth, Germany). Chocolate cake with a gluten concentration of 200 mg/kg (stock chocolate cake) was produced as described, except that cake mix containing wheat flour cv. Genius was used. The amount of wheat flour in the cake mix (275 mg gluten/kg) was adjusted to provide a final gluten concentration of 200 mg/kg in the chocolate cake.

Gluten-containing chocolate cake samples for the study were prepared as follows: 10 mg/kg, 25 g stock chocolate cake was mixed with 475 g gluten-free chocolate cake; 20 mg/kg, 50 g stock chocolate cake was mixed with 450 g gluten-free chocolate cake; and 100 mg/kg, 250 g stock chocolate cake was mixed with 250 g gluten-free chocolate cake. Mixtures were shaken in an overhead shaker for at least 1 h.

The rice-based crisp bread represented a more heavily heat-treated sample. Gluten-free crisp bread was prepared by mixing 270 g of gluten-free rice flour (*see* above) and 2.7 g NaCl with 270 mL of ice-cold water using a hand mixer at high speed (air incorporation). The mass was distributed in two round baking tins (25 cm diameter) to yield a dough layer of approximately 1 cm. The dough surface was perforated with a needle, and the dough was baked at 230°C for 30 min, then turned upside down and baked for another 30 min. After cooling overnight, the bread was lyophilized and ground to a fine powder using a mortar and pestle. Crisp bread containing 200 mg gluten/kg (stock crisp bread) was produced as described, except that gluten-containing stock rice flour (200 mg gluten/kg, *see* above) was used.

Gluten-containing crisp bread samples for the study were prepared as follows: 10 mg/kg: 17.5 g stock crisp bread was mixed with 332.5 g gluten-free crisp bread; 20 mg/kg, 35 g stock crisp bread was mixed with 315 g gluten-free crisp bread; and 100 mg/kg, 175 g stock crisp bread was mixed with 175 g gluten-free crisp bread. Mixtures were shaken in an overhead shaker for at least 1 h.

The analyses of homogeneity (*see* below) revealed that the gluten-free crisp bread was contaminated with gluten at a very low concentration of about 4.5 mg gluten/kg. This may have happened during production of the crisp breads, in particular during the grinding and sifting steps. Therefore, the target gluten concentrations of the crisp bread samples (0, 10, 20, and 100 mg/kg) were corrected to the gluten concentrations that were in fact present (4.5, 15, 24, and 102 mg/kg).

Homogeneity of Samples

All samples were checked for homogeneity before they were packaged in airtight bottles and accepted for the collaborative study. This was done by taking 10 representative 1 g aliquots from each bulk sample and then analyzing by the G12 Sandwich ELISA. Ideally, the CV of the 10 determinations should be 15% or less. Most samples with gluten concentration above 4 mg/kg complied with this, except the chocolate cake samples containing a low concentration (≤20 mg/kg) of incurred gluten showed a CV of 21%. This was considered allowable for a sample like chocolate cake containing below 20 mg/kg gluten, because in an earlier study a beer (>20 mg/kg) and a starch syrup (<20 mg /kg) sample were accepted with a CV of 18–22% (7, 8).

Shipment

Two independent blinded replicates for each sample were provided to the participating laboratories. The coded sample vials contained 1 g of sample. Samples were shipped together with ELISA kits, instructions, and result sheet to participating laboratories.

Analysis and Data Reporting

Participants were requested to follow the instructions and to extract each sample using the test kit's standard procedure and to analyze in duplicate in one analytical run. If changes had been made to the analytical protocol, they had to be reported in the "comments" box of the result sheet. The samples were analyzed

Table 5. Lot-to-lot variation (reproducibility): three different kit batches of the AgraQuant Gluten G12 test kit were run, GU1001-1106, GU1002-1108, and GU1003-1111. Mean OD values, SD, and CV are shown below. All CV values for interbatch analysis were 15% or less, meeting the manufacturer's QC criteria

Standard, mg/kg	GU1001-1106	GU1002-1108	GU1003-1111	Mean (OD)	SD (OD)	CV, %
0	0.10	0.10	0.09	0.10	0.01	6.97
4	0.26	0.21	0.21	0.23	0.03	11.55
20	0.72	0.57	0.58	0.63	0.08	13.45
80	1.39	1.13	1.09	1.21	0.16	13.65
200	2.11	1.65	1.60	1.79	0.28	15.92

Table 6. Samples from FAPAS Proficiency Test 2781 (February 2011), 2792 (June 2011), and 2795 (October 2011) were analyzed during the single-laboratory validation by Romer Labs using the AgraQuant Gluten G12 test kit. Test materials from Round 2781 were cake mix to be analyzed for gluten. Test materials were prepared using a gluten and wheat free chocolate cake mix, to which a gluten and wheat containing cake mix was added. Test materials from Round 2792 were prepared by mixing infant soya formula with wheat flour. Test materials from Round 2795 were prepared by combining cake mix with wheat flour. Analysis of the FAPAS 2781, 2792, and 2795 proficiency samples using the AgraQuant Gluten G12 test kit produced very similar results to those assigned values for the R5 Mendez method (data from the R-Biopharm kit). The R5 Mendez method is currently the Codex Type I approved method for gluten analysis (4)

		Assigned value		
	AgraQuant Gluten G12 test kit, mg/kg gluten	R5 ELISA–R-Biopharm R7001, mg/kg gluten	Veratox ELISA–Neogen, mg/kg gluten	
FAPAS 2781 A	<4	Negative	Negative	
FAPAS 2781 B	22.6	27.4	42.6	
FAPAS 2781 C	95.6	91.6	120.7	
		R5 ELISA–R-Biopharm R7001, mg/kg gluten	AR5 ELISA–R-Biopharm R7002, mg/kg gluten	
FAPAS 2792 A	119.8	134.2	141.0	
FAPAS 2792 B	<4	Negative	Negative	
		R5 ELISA–R-Biopharm R7001, mg/kg gluten	R5 ELISA–R-Biopharm R7002, mg/kg gluten	Assigned value Ingenasa–R5 ELISA 30.GLU.K2, mg/kg gluten
FAPAS 2795 A	51.3	58.5	43.4	71.4
FAPAS 2795 B	<4	Negative	Negative	Negative

by each laboratory. All optical density (OD) values had to be recorded in a ready-to-use Excel sheet. The participants used the calculator, which was provided with the Excel sheet. The model was a simple linear point-to-point calculation. The final data from the laboratories were sent to the study coordinator. A statistical evaluation was performed according to AOAC guidelines (9, 10).

AOAC Official Method 2014.03
Gluten in Rice Flour and Rice-Based Food Products
G12 Sandwich ELISA
First Action 2014

(Applicable for determination of gluten in rice flour and rice-based unprocessed and processed foods as evaluated in the multilaboratory study.)

Caution: Wear protective gloves and safety glasses. The stop solution contains acid. Avoid contact with skin or eyes. If exposed, flush with water (*see* Material Safety Data Sheet). The extraction solution contains chemicals which are harmful to health. Perform sample extraction under a chemical hood and avoid contact with skin. Dispose of all materials, containers, and devices appropriately after use.

See Table **2014.03A** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

The method is based on an enzyme immunoassay format using a monoclonal G12 antibody that can determine gluten derived from wheat, rye, barley, and cross-bred varieties. The G12 antibody binds to the celiac toxic amino acid sequence

Table 2014.03A. Performance statistics for the overall G12 sandwich ELISA results

		Sample ID[a]											
Parameter	Symbol	1	2	3	4	5	6	7	8	9	10	11	12
Total No. laboratories	*P*	17	18	18	18	16	18	18	16	17	18	18	18
Total No. replicates	Sum [n(L)]	34	36	36	36	32	36	36	32	34	36	36	36
Overall mean of all data (grand mean; mg/kg)	xbarbar	1.6	13.5	26.2	101.2	0.1	6.2	13.1	63.5	4.1	14.9	26.6	112.7
Repeatability SD, mg/kg	s_r	0.8	2.5	8.1	14.8	1.2	1.2	1.3	5.1	1.9	1.5	4.3	20.4
Reproducibility SD, mg/kg	s_R	1.9	4.0	11.6	31.8	1.2	1.8	2.5	13.5	2.8	4.5	8.9	33.2
Repeatability RSD, %	RSD_r	48.2	18.5	30.7	14.7	2348	19.2	10.2	8.0	46.2	10.4	16.2	18.1
Reproducibility RSD, %	RSD_R	115.8	29.6	44.2	31.4	2348	28.3	19.1	21.2	69.0	30.3	33.6	29.4
Bias (mg/kg) observed-nominal		1.6	3.5	6.2	1.2	0.1	–3.8	–6.9	–36.5	–0.4	–0.1	2.6	10.7
Recovery, % = observed/nominal × 100			135.0	131.0	101.2		62.0	65.5	63.5	91.1	99.3	110.8	110.5

[a] 1 = Gluten-free rice flour; 2 = rice flour 10 mg gluten/kg; 3 = rice flour 20 mg gluten/kg; 4 = rice flour 100 mg gluten/kg; 5 = gluten-free chocolate cake; 6 = chocolate cake 10 mg gluten/kg; 7 = chocolate cake 20 mg gluten/kg; 8 = chocolate cake 100 mg gluten/kg; 9 = crisp bread 4.5 mg gluten/kg; 10 = crisp bread 15 mg gluten/kg; 11 = crisp bread 24 mg gluten/kg; and 12 = crisp bread 102 mg gluten/kg.

Table 2014.03B. Cross-reactivity of the G12 antibody (G12 antibody shows no cross-reactivity to various nuts, oils, seeds, starches, or gluten-free grains)

Food category	Food sample	Romer extraction solution, mg/kg gluten	Gluten, %
Gluten-containing grains	Wheat flour	72222	7.2
	Barley (Cumion)	292390	29.2
	Durum wheat	15733	1.6
	Spelt (Ostro)	81926	8.2
	Rye (Capitan)	41577	4.2
Naturally gluten-free grains	Soya bean	<4	
	Soya mince	<4	
	Buckwheat	<4	
	Rice flour	<4	
	Quinoa	<4	
	Corn kernels	<4	
	Teff flour	<4	
	Millet	<4	
Oats	Bastion	4.3	
	00-61 Cn	7.4	
	Brachan	<4	
	Husky	6.3	
	Fusion	6.6	
Nuts	Pecan	<4	
	Walnut	<4	
	Almond	<4	
	Cashew	<4	
	Macadamia	<4	
	Peanut	<4	
	Hazelnut	<4	
	Pine nut	<4	
	Pistachio	<4	
Seeds	Golden linseed	<4	
	Brown linseed	<4	
	Poppy	<4	
	Sesame	<4	
	Mustard	<4	
Oils	Hazelnut oil	<4	
	Walnut oil	<4	
	Vegetable oil	<4	
	Sunflower oil	<4	
Starches	Tapioca starch	<4	
	Wheat starch	<4	
	Potato starch	<4	
Miscellaneous	Amaranth	<4	

QPQLPY and related sequences in rye and barley. The antibody detects prolamins in nonheated and heated food by using a specific proprietary extraction solution. No cross-reactivity has been determined to maize, rice, teff, millet, buckwheat, quinoa, amaranth, and soy (*see* Table **2014.03B**).

Gluten is extracted from samples using proprietary extraction solution containing reducing agents followed by ethanol extraction. After centrifugation the supernatant is used in a sandwich enzyme-linked immunoassay. When incubated on monoclonal antibody-coated microwells, the analyte is forming an antibody-antigen complex. After a washing step, an enzyme-conjugated monoclonal antibody is applied to the well and incubated. After a second washing step, an enzyme substrate is added and blue color develops. The intensity of the color is directly proportional to the concentration of gluten in the sample or standard. A stop solution is then added which changes the color from blue to yellow. The microwells are measured optically using a microwell reader with a primary absorbance filter of 450 nm (OD450). The optical densities of the samples are compared to the standards and an interpolated result is determined.

B. Apparatus

The apparatus specified has been tested. Equivalent apparatus may be used.

(**a**) *Osterizer blender*.—Used for homogenization of sample (Sunbeam-Oster, Ft. Lauderdale, FL).

(**b**) *Centrifuge tubes*.—50 mL for extraction (Star Labs International GmbH, Hamburg, Germany).

(**c**) *Glassware*.—Wash bottle (1000 mL) and graduated cylinders.

(**d**) *Water bath*.—Grant Sub Aqua 12 (Grant Instruments, Cambridgeshire, UK).

(**e**) *Stuart roller mixer*.—Bibby Scientific Ltd (Staffordshire, UK).

(**f**) *Bench top centrifuge*.—Sigma 1-14 (Sigma Laborzentrifugen, Osterode am Harz, Germany).

(**g**) *Centrifuge tubes*.—2 mL; for sample dilution (Star Labs International GmbH).

(**h**) *Micropipet*.—Accurately delivering 100 µL ± 1%.

(**i**) *Microtiter plate reader with a 450 nm filter*.—Thermo Fisher Scientific (Shanghai, China).

C. Reagents

The following items (**a**)–(**i**) are available as a test kit (AgraQuant Gluten G12 ELISA®, Romer Labs UK Ltd, Runcorn, UK). All reagents are stable for 12 months from date of manufacture at 2–8°C (36–46°F). Refer to kit label for current expiration.

(**a**) *Antibody-coated microwell strips*.—Monoclonal antibodies are coated in 20 mM phosphate buffered saline (PBS) onto a set of 12 eight-microwell strips (NUNC, Roskilde, Denmark).

(**b**) *Gluten ready-to-use standards (antigen)*.—Five vials containing 1.2 mL of each gluten G12 standard (0, 4, 20, 80, and 200 mg/kg labeled as ppm), prepared by vital wheat gluten dissolved in 60% ethanol at a concentration of 1 mg/mL. Solution is further diluted in 20 mM PBS–Tween (0.9% sodium chloride, 0.07% Tween 80) containing 0.25% fish gelatin (Sigma) to 0,

10, 50, 200 and 500 ng/mL gluten, calibrated to the WGPAT gliadin (86% highly purified gliadin from 40 different European wheat varieties).

(**c**) *Conjugate solution (peroxidase-labeled antibody, ready-to-use)*.—One bottle containing 13 mL.

(**d**) *Substrate solution (stabilized peroxide substrate and 3,3',5,5'-tetramethyl-benzidine in a dilute buffer solution)*.—One bottle containing 15 mL.

(**e**) *Stop solution (1 N H_2SO_4)*.—One bottle containing 15 mL.

(**f**) *Diluent buffer*.—One bottle containing 20 mL of 5× concentrated diluent buffer. Contains a final concentration of 20 mM PBS-Tween (0.9% sodium chloride, 0.07% Tween 80) with 0.25% fish gelatin (Sigma) and 0.01% Proclin as a preservative.

(**g**) *Wash buffer*.—One bottle containing 60 mL of 10× concentrated wash buffer. Contains a final concentration of 20 mM PBS-Tween (0.9% sodium chloride, 0.05% Tween 20) with 0.01% Proclin as preservative.

(**h**) *Extraction solution*.—One bottle containing 105 mL of ready-to-use proprietary extraction solution containing reducing agents.

(**i**) *Fish gelatin*.—One sachet containing 10 g.

Additional reagents needed, but not provided with the test kit:

(**a**) *Distilled or deionized water*.

(**b**) *Ethanol*.—80% (v/v).

D. General Instructions

Due to the sensitivity of the assay, a gluten-free environment must be maintained. It is preferable to perform the assay in a separate room from that used for sample preparation and extraction. Make sure balance and the surrounding space, as well as equipment such as spatulas, are clean. Cleaning can be done by using a 70% alcoholic solution. Spatula should be cleaned after each sample weighing by a 70% alcoholic solution.

Store kit at 2–8°C (35–46°F) and let all components equilibrate to 20–25°C (68–77°F) before use.

Include ready-to-use standards in duplicates to each run of samples. Use separate pipet tips for each standard and each sample extract to avoid cross-contamination.

It is recommended that an eight-channel pipettor is used to perform the assay. No more than 48 samples and standards total should be run in one experiment when using an eight-channel pipettor (24 when samples and standards are added in duplicate, e.g., six test strips). If using only single-channel pipets, it is recommended that no more than a total of 16 samples and standards are analyzed in one experiment (eight when standards and samples are added in duplicate, e.g., two test strips).

E. Preparation of Components Delivered with the Kit

(**a**) *Sample dilution buffer*.—Dilute diluent buffer concentrate 1:5 with distilled water (e.g., add 20 mL of concentrated diluent buffer to 80 mL distilled water). Dilution buffer may be used within 24 h, if stored at 4°C.

(**b**) *Wash buffer*.—If a precipitate is formed during storage of the wash buffer concentrate, the concentrate should be warmed up until it is dissolved. Dilute wash buffer concentrate 1:10 with distilled water (e.g., add 10 mL of concentrated wash buffer to 90 mL distilled water). Wash buffer may be used within 1 week, if stored at 4°C.

F. Sample and Test Portion Preparation

Obtain a representative sample and homogenize a minimum of 5 g in a mortar or blender as fine as possible. Weigh out 0.25 g of homogenized sample into a vial with a minimum 10 mL capacity, which can be tightly sealed. For chocolate-containing samples, additionally add 0.25 g of powdered fish gelatin. Add 2.5 mL extraction solution (under a fume/chemical hood), close vials, and mix vigorously on a vortex. Visually check for clumps, and continue mixing until samples are well dispersed in the extraction solution.

Incubate at 50°C (122°F) for 40 min in a water bath. Allow the extracts to cool to room temperature and add 7.5 mL of 80% ethanol; mix well. Shake for a total of 60 min at room temperature (20–25°C/68–77°F) with a rotary shaker. (After about 30 min in the rotator, check the vials visually if all sample material has suspended in the liquid. If clumps have formed, vortex and let the vials rotate for the second 30 min to complete the extraction procedure).

Centrifuge samples for 10 min at 2000 × *g* to obtain a clear aqueous layer between the particulate sediment and supernatant. Note, in some cases, a thin fatty layer creaming on top of the supernatant. Collect the aqueous supernatant (extract) and transfer into a new vial. Dilute supernatant at least 1:10 (0.1 + 0.9 mL) with prediluted sample dilution buffer (depending on the expected prolamin content of the sample). If prediluted samples are not immediately used for determination by ELISA, close vials and keep in the dark at room temperature (20–25°C/68–77°F) for a maximum of 7 days until ELISA experiments.

G. Determination (Assay)

Bring all reagents to room temperature (20–25°C, 68–77°F) before use.

Use dilution of the sample extract to carry out ELISA experiments. Run standards and diluted sample extracts in duplicate. Place an appropriate number of antibody-coated microwells in a microwell strip holder. Record standard and sample positions.

Using a single-channel pipettor, add 100 µL of each ready-to-use standard or prepared sample into the appropriate well. Use a fresh pipet tip for each standard or sample. Make sure the pipet tip has been completely emptied.

Incubate at room temperature (20–25°C, 68–77°F) for 20 min. Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with diluted wash buffer, and then emptying the buffer from the microwell strips. Repeat this step four times for a total of five washes. Take care not to dislodge the strips from the holder during the wash procedure. Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel all of the residual buffer after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

Measure the required amount of conjugate from the green-capped bottle (about 120 µL/well or 1 mL/strip) and place in a separate container (e.g., reagent boat when using the

eight-channel pipettor). Using an eight-channel pipet, dispense 100 µL of conjugate into each well.

Incubate at room temperature (20–25°C, 68–77°F) for 20 min. Empty the contents of the microwell strips into a waste container. Wash by filling each microwell with diluted wash buffer, and then emptying the buffer from the microwell strips. Repeat this step four times for a total of five washes. Take care not to dislodge the strips from the holder during the wash procedure. Lay several layers of absorbent paper towels on a flat surface and tap microwell strips on towels to expel all of the residual buffer after the fifth wash. Dry the bottom of the microwells with a dry cloth or towel.

Measure the required amount of substrate from the blue-capped bottle (about 120 µL/well or 1 mL/strip) and dispense into a separate container (e.g., reagent boat for an eight-channel pipettor).

Pipet 100 µL of the substrate into each microwell using an eight-channel pipettor. Incubate at room temperature (20–25°C, 68–77°F) for 20 min in the dark.

Measure the required amount of stop solution from the red-capped bottle (about 120 µL/well or 1 mL/strip) and dispense into a separate container (e.g., reagent boat for an eight-channel pipet).

Pipet 100 µL of stop solution into each microwell using an eight-channel pipettor. The color should change from blue to yellow.

H. Reading

Eliminate air bubbles prior to reading wells as they are likely to affect analytical results.

Read the absorbance of wells with a microwell reader using a 450 nm filter. Record OD readings for each microwell.

I. Calculations

Use unmodified OD values or OD values expressed as a percentage of the OD of the 200 ppm standard to construct a dose-response curve using the five standards (0, 4, 20, 40, and 200 ppm gluten). Gluten concentration given for the standards already consider sample preparation and 1:10 dilution according to method protocol. Gluten concentrations of samples can be calculated by interpolation from this standard curve using a point-to-point calculation.

If a sample contains gluten levels higher than the highest standard (>200 ppm), the sample extract should be further diluted with dilution buffer such that the diluted sample results are in the range of 4 to 200 ppm and reanalyzed to obtain accurate results. The dilution factor must be included when the final result is calculated.

J. Criteria for Acceptance of Standard Curve

An example for the calibration curve is shown in the Certificate of Analysis included in each test kit. Higher OD values of the absorbance at 450 nm compared to the certificate may indicate insufficient washing or gluten contamination. For samples showing OD values higher than the 200 ppm standard, a further dilution and repeated analysis is recommended. The additional dilution factor must be taken into consideration during calculation.

Any coloration of the substrate solution prior to the analysis or OD value of less than 1.1 absorbance units for 200 ppm standard may indicate instability or deterioration of reagents.

Collaborator´s Comments

Participants were following the instructions and the study coordinator did not receive any comments that changes to the procedure had been made. One laboratory reported that the test kit was not cold on arrival, but the results could still be used.

Results and Discussion

Two laboratories returned result sheets that could not be used. This was due to high CV in calibration duplicates and incomplete result sheets. Negative results that were reported <LOD in the Excel calculator sheet were calculated by a linear back-extrapolation method using a linear regression curve fit for lower calibrators (0, 4, and 20 mg/kg).

Finally, the results from 18 laboratories were used for the evaluation (*see* Table 7). Outliers were identified by using the Cochran and the Grubbs tests according to AOAC guidelines (9). After removal of the outliers, the statistical performance was calculated. The results of the calculations are shown in Table **2014.03A**.

The LOD was calculated according to recommendations from AOAC (9, 10). A plot of the reproducibility SD (s_R) versus the mean for all samples ($\bar{x}$) in the dataset was created (*see* Figure 2). With this plot the LOD was calculated using the intercept of the linear regression line, which was 0.69. Using slope correction, this resulted in a calculated s_0 of 1.30 mg/kg. Since LOD = 3.3 × s_0, the LOD of the method was 4.3 mg/kg. The RSDs were between 20 and 30% for most of the gluten containing samples. The RSD_R was in a similar range as found for other ELISA methods (7, 8). The contaminated crisp bread had a higher RSD_R, but the trace of 4.5 mg/kg was close to the LOD of the method, hence a higher RSD could be expected (11). Overall, the G12 method was able to detect and quantify low gluten concentrations in these different matrixes.

According to Abbott et al. (10), recoveries between 80 and 120% are ideal for ELISA methods. Recoveries in a range between 50 and 150% are acceptable as the extended recovery range for incurred samples or difficult matrixes. For the present study, the spiked rice flour showed a recovery range of 101–135%, and the recovery for the rice-based crisp bread was 91–111%. For low levels of spiked gluten at 10 mg/kg, the G12 method is sensitive to a gluten spike with average recovery of 130%. With the gluten-incurred chocolate cake the recovery was 62–66%, which is at the lower end of acceptable recovery. Details for recoveries and biases per matrix of individual concentrations are shown in Table **2014.03A**.

The chocolate cake recipe contained eggs, fat, chocolate, and hydrocolloid (guar gum). Ingredients like egg proteins are strong thermal aggregators possibly resulting in highly insoluble covalently bonded (S-S) aggregates with incorporated gluten proteins. In general, the reducing agent in the ELISA extraction medium can usually deal with heat-aggregated gluten. The high fat content of more than 20% based on dry mass as well as the presence of polyphenols from chocolate might have promoted interactions with gluten proteins affecting gluten recovery. Furthermore, guar gum acted as a thickener during extraction

Table 7. Individual results from interlaboratory study for gluten by AgraQuant Gluten G12 Sandwich ELISA (results are stated as mg gluten/kg). Outliers determined with Cochran and the Grubbs test are marked in bold font and highlighted in grey. Laboratory B did not return a result sheet in time. Result sheets of laboratories C and J could not be used

	Sample ID[a]																							
Lab	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Sample 7		Sample 8		Sample 9		Sample 10		Sample 11		Sample 12	
A	0, 7	0, 3	14, 1	18, 3	21, 4	23, 1	107, 5	115, 5	–1, 1	0, 1	4, 6	4, 7	12, 4	10, 1	61, 0	59, 5	2, 5	3, 2	12, 0	13, 7	26, 5	24, 9	92, 1	109, 9
D	1, 1	3, 6	18, 5	17, 6	39, 8	53, 9	150, 5	162, 8	0, 9	0, 3	9, 3	7, 1	16, 2	17, 3	121.4[b]	120.7[b]	5, 8	3, 7	16, 8	18, 2	45, 0	41, 6	154, 5	159, 7
E	0, 4	0, 6	17, 7	14, 7	19, 1	48, 6	149, 1	177, 6	0, 9	–1, 2	6, 7	6, 1	12, 6	15, 9	79, 7	78, 5	0, 3	0, 5	11, 8	11, 0	17, 8	19, 9	102, 0	104, 9
F	0, 2	0, 4	11, 4	15, 6	19, 2	10, 2	58, 1	71, 2	0, 0	–2, 1	5, 3	5, 4	12, 1	10, 9	71, 2	62, 3	6, 7	2, 0	12, 0	9, 1	18, 8	18, 4	86, 7	79, 3
G	1, 2	0, 8	9, 1	14, 6	27, 3	26, 3	76, 7	70, 4	0, 5	–0, 9	7, 2	4, 9	12, 0	11, 6	52, 2	55, 4	4, 0	3, 0	13, 5	13, 8	22, 2	27, 0	103, 6	86, 5
H	–0, 2	–0, 3	7, 7	7, 2	12, 6	14, 1	64, 2	60, 8	0, 7	0, 3	5, 3	6, 1	11, 9	13, 1	60, 4	59, 9	0, 7	0, 6	9, 1	8, 9	17, 3	16, 4	73, 8	66, 3
I	2, 0	2, 8	15, 6	12, 9	22, 7	19, 0	81, 5	78, 0	–1, 3	1, 9	3, 9	7, 1	10, 1	11, 7	51, 1	39, 0	5, 5	4, 9	15, 2	15, 0	39, 3	26, 2	65, 8	100, 4
K	6, 7	5, 6	15, 7	15, 5	38, 7	29, 9	85, 6	76, 0	0, 4	–0, 8	4, 3	4, 5	8, 9	11, 2	40, 1	39, 4	13.5[b]	14.6[b]	26, 0	23, 3	36, 4	36, 8	158, 4	114, 6
L	8.9[c]	0.5[c]	22, 0	17, 5	38, 3	56, 8	120, 5	143, 2	2, 9	–1, 7	9, 8	9, 1	12, 9	15, 1	66, 2	75, 4	3, 0	4, 7	13, 4	17, 2	34, 9	21, 4	118, 5	175, 7
M	3, 9	3, 5	18, 5	17, 7	20, 3	38, 9	99, 6	81, 4	0, 6	–0, 4	5, 3	7, 6	15, 5	13, 6	70, 3	78, 8	6, 9	11, 1	21, 3	19, 7	16, 9	21, 1	154, 6	90, 3
N	1, 7	4, 7	9, 7	12, 7	18, 9	15, 0	145, 0	111, 6	0, 1	0, 9	4, 5	3,8	9,6	9,5	45,5	52,0	4,8	10,9	11,1	10,4	20,6	23,8	102,1	90,5
O	1,3	1,9	15, 9	7, 4	26, 2	36, 2	124, 5	95, 6	0.3[c]	1.5[c]	6, 8	4, 9	18, 7	13, 9	73.6[c]	108.7[c]	4, 4	1, 6	19, 3	15, 3	26, 7	27, 5	144, 8	142, 8
P	–0, 6	1, 1	7, 7	11, 1	14, 5	14, 2	74, 1	74, 5	0, 5	0, 1	7, 5	7, 0	13, 7	12, 7	71, 3	66, 1	6, 6	3, 5	12, 0	16, 4	22, 9	18, 7	75, 7	70, 8
Q	0, 3	0, 6	12, 6	10, 3	18, 9	13, 7	114, 9	108, 1	–0, 7	–1, 2	5, 3	6, 6	12, 1	13, 2	67, 7	69, 7	2, 5	2, 3	12, 6	11, 3	23, 2	20, 3	112, 9	99, 8
R	3, 3	3, 6	11, 3	15, 2	25, 2	32, 2	139, 4	93, 1	0, 3	0, 1	6, 5	6, 4	14, 1	12, 8	72, 6	70, 5	6, 4	6, 6	19, 3	19, 1	46, 0	38, 9	178, 5	152, 9
S	0, 8	1, 0	9, 9	7, 6	31, 9	17, 9	92, 3	58, 7	0, 9	0, 3	5, 7	3, 8	10, 8	10, 3	44, 1	55, 1	1, 3	2, 0	12, 4	11, 9	28, 6	18, 2	109, 2	60, 7
T	–1, 1	–1, 4	10, 1	9, 3	21, 0	22, 7	95, 2	77, 8	0, 2	0, 1	6, 6	4, 6	14, 2	14, 2	71, 8	76, 9	1, 7	1, 1	9, 8	10, 6	19, 8	20, 0	109, 7	129, 0
U	2, 5	2, 8	16, 4	17, 7	26, 5	29, 0	97, 0	109, 4	–2.28[b]	–2.74[b]	11, 0	7, 8	17, 3	17, 9	76, 9	91, 2	4, 6	8, 4	19, 6	22, 7	33, 4	41, 4	139, 6	141, 3

[a] 1 = Gluten-free rice flour; 2 = rice flour 10 mg gluten/kg; 3 = rice flour 20 mg gluten/kg; 4 = rice flour 100 mg gluten/kg; 5 = gluten-free chocolate cake; 6 = chocolate cake 10 mg gluten/kg; 7 = chocolate cake 20 mg gluten/kg; 8 = chocolate cake 100 mg gluten/kg; 9 = crisp bread 4.5 mg gluten/kg; 10 = crisp bread 15 mg gluten/kg; 11 = crisp bread 24 mg gluten/kg; and 12 = crisp bread 102 mg gluten/kg.

[b] Grubbs outlier.

[c] Cochran outlier.

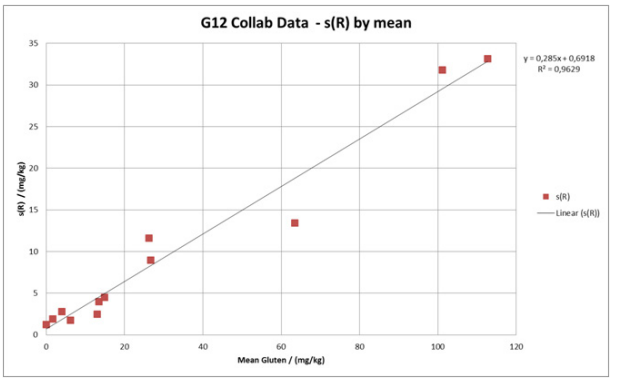

Figure 2. Plot of reproducibility SD (S_R) versus the global mean observed gluten concentration for the interlaboratory study.

and strongly increased the viscosity of the extract. Hence, a clear separation of extract aliquots was more difficult with this matrix. This may also explain the higher CV in the homogeneity tests. Due to the complexity of the cake recipe we cannot pinpoint a single reason for low recovery, but a combination of the factors mentioned is most likely. Taking this complexity into account, the method evaluated here largely complies with the guidelines and best practices for allergen ELISA methods (10). With an LOD of 4.3 mg gluten/kg, it fulfills the LOD requirement of ≤10 mg/kg of Codex Alimentarius (4).

Conclusions and Recommendation

This collaborative study has shown that the G12 Sandwich ELISA is capable of quantifying gluten in foods with an LOD of 4.3 mg gluten/kg. This method shows good precision and accuracy in the concentration range of most interest (20 mg/kg and above), where it has to be decided whether a sample meets guidelines for gluten content. Some matrix effects, especially with the incurred chocolate cake samples, may lower recovery as compared to spiked samples. Therefore, it may be beneficial to occasionally check recovery by using internal reference samples with known gluten content.

According to these results, it is recommended that the method be accepted by AOAC as *Official First Action*.

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Expert Review Panel on Food Allergens - Gluten

OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final revised version of the Official Chair's Report for the Expert Review Panel on Food Allergens - Gluten held on March 20, 2014.

Shang-Jing (Jean) Pan, Expert Review Panel Chair

4/7/2014
Date

Please sign, date and fax this document to La'Kia Phillips at 301-924-7089.

METHODS FOR CONSIDERATION

Conclusion: The Expert Review Panel reviewed the collaborative study for **OMAMAN-09: Detection Of Gluten In Food By Enzyme Immunoassay Method Based On A Specific Monoclonal G12 Antibody To The Celiac Toxic Amino Acid Prolamin Sequences**. **Methods Reviewed:** Each method collected by AOAC for consideration by this ERP is reviewed by all members. The decisions of this ERP are reflective of both the submitted method review forms and the in person meeting held on Thursday, March 20, 2014.

METHOD NO.	MANUSCRIPT TITLE
OMAMAN-09	**DETECTION OF GLUTEN IN FOOD BY ENZYME IMMUNOASSAY METHOD BASED ON A SPECIFIC MONOCLONAL G12 ANTIBODY TO THE CELIAC TOXIC AMINO ACID PROLAMIN SEQUENCES: COLLABORATIVE STUDY** *AUTHORS* Elisabeth Halbmayr-Jech, Adrian Rogers, Clyde Don, Michael Prinster, Romer Labs Division Holding GmbH, Technopark 1, 3430 Tulln, Austria, Romer Labs UK Ltd, Block 5, The Heath Technical & Business Park, Runcorn, Cheshire WA7 4QX, United Kingdom, Foodphysica, Vogelwikke 12, 6665 HP Driel, The Netherlands, Romer Labs Inc, 1301 Stylemaster Drive, Union, MO 63084-1156, USA *COLLABORATORS* G. Augustin, C. Brewe, Z. Bugyi, S. Tomoszi, D. Clarke, P. Cressey, A. Firzinger, J. Gelroth, M. Hemingway, R. Hochegger, J. Jolly, P. Kasturi, P. Koehler, C. Poirier, T. Koerner, A. Rogers, G. Sharma, R. Sherlock, C. Sousa, S. Taylor, J. Topping, P. Wehling, M. Marquard

ERP DECISION(S)	ERP ACTIONS FOR OTHER & FINAL ACTION REQUIREMENTS	VOTE	DECISION DATE
Motion to move forward to First Action Official Methods status based upon the revisions to the manuscript and the supplemental information.	*N/A*	MOTION PASSED ***UNANIMOUS*** *Wheling, Garber*	March 20, 2014

EXPERT REVIEW PANEL MEMBERS

Shang-Jing Pan, Abbott Nutrition
Sneh Bhandari, Silliker, Inc.
Joe Boison, Canadian Food Inspection Agency
Eric Garber, US FDA
Todd Marrow, University of Guelph
Girdhari Sharma, FDA
Paul Wehling, General Mills, Inc.

Not Present

Bert Popping, Eurofins Scientific, Inc.
Terry Koerner, Health Canada

AOAC STAFF

Jim Bradford, Executive Director
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Deborah McKenzie
La'Kia Phillips

OBSERVERS

Michael Prinster, Romer Labs

Below are the noted reviewers for OMAMAN-09. Expert Review Panel members are required to review the method for discussion.

Reviewer	
Joe Boison	Primary Reviewer
Todd Marrow	Secondary Reviewer
Julie Drotz	Safety Reviewer
Sidney Sudberg*	Statistical Reviewer

**Statistical Review was not completed as assigned. Paul Wehling completed the review on behalf of Sidney Sudberg.*

AOAC Official Method 2014.01
Salmonella in Selected Foods
3M™ Petrifilm™ *Salmonella* Express System
First Action 2014

[Applicable to detection of *Salmonella* spp. in raw ground beef (25 g), raw ground chicken (25 g), pasteurized liquid whole egg (100 g), raw ground pork (25 g), cooked chicken nuggets (325 g), frozen uncooked shrimp (25 g), fresh bunched spinach (25 g), dry dog food (375 g), and stainless steel. Not applicable to some lactose-positive *Salmonella* species.]

See Tables **2014.01A** and **B** for results of the interlaboratory study supporting acceptance of the method. *See* Appendix available on the *J. AOAC Int*. website for detailed tables of results of the collaborative study (http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

Caution: Do not use the 3M Petrifilm SALX System method in the diagnosis of conditions in humans or animals. To reduce the risks associated with exposure to chemicals and biohazards, perform pathogen testing in a properly equipped laboratory under the control of trained personnel. Always follow standard good laboratory safety practices (GLP), including proper containment procedures, and wearing appropriate protective apparel and eye protection while handling testing materials and test samples. Avoid direct contact with the contents of the enrichment medium and inoculated plates. Dispose of enrichment media and inoculated plates according to all applicable government regulatory regulations and applicable laboratory procedures. Wear appropriate protective apparel while handling the 3M Petrifilm SALX Plate as some of the components may be considered allergenic and irritants to some individuals.

Table 2014.01A. Summary of results for detection of *Salmonella* in raw ground beef (25 g)

Method[a]	3M Petrifilm *Salmonella* Express System with alternative confirmation			3M Petrifilm *Salmonella* Express System with traditional confirmation		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. of samples analyzed	2/168	85/168	168/168	2/168	85/168	168/168
Candidate presumptive POD (CP)	0.01 (0.00, 0.04)	0.51 (0.43, 0.58)	1.00 (0.98, 1.00)	0.01 (0.00, 0.04)	0.51 (0.43, 0.58)	1.00 (0.98, 1.00)
s_r[b]	0.11 (0.10, 0.15)	0.51 (0.46, 0.52)	0.00 (0.00, 0.15)	0.11 (0.10, 0.15)	0.51 (0.46, 0.52)	0.00 (0.00, 0.15)
s_L[c]	0.00 (0.00, 0.04)	0.00 (0.00, 0.13)	0.00 (0.00, 0.15)	0.00 (0.00, 0.04)	0.00 (0.00, 0.13)	0.00 (0.00, 0.15)
s_R[d]	0.11 (0.10, 0.12)	0.51 (0.47, 0.52)	0.00 (0.00, 0.21)	0.11 (0.10, 0.12)	0.51 (0.47, 0.52)	0.00 (0.00, 0.21)
P-value[e]	0.5158	0.9341	1.0000	0.5158	0.9341	1.0000
Candidate confirmed positive/ total No. of samples analyzed	0/168	83/168	168/168	1/168	83/168	168/168
Candidate confirmed POD (CC)	0.00 (0.00, 0.02)	0.49 (0.42, 0.57)	1.00 (0.98, 1.00)	0.01 (0.00, 0.03)	0.49 (0.42, 0.57)	1.00 (0.98, 1.00)
s_r	0.00 (0.00, 0.15)	0.51 (0.46, 0.52)	0.00 (0.00, 0.15)	0.08 (0.07, 0.15)	0.51 (0.46, 0.52)	0.00 (0.00, 0.15)
s_L	0.00 (0.00, 0.15)	0.00 (0.00, 0.11)	0.00 (0.00, 0.15)	0.00 (0.00, 0.03)	0.00 (0.00, 0.11)	0.00 (0.00, 0.15)
s_R	0.00 (0.00, 0.21)	0.51 (0.47, 0.52)	0.00 (0.00, 0.21)	0.08 (0.07, 0.09)	0.51 (0.47, 0.52)	0.00 (0.00, 0.21)
P-value	1.0000	0.9757	1.0000	0.4418	0.9757	1.0000
Positive reference samples/ total No. of samples analyzed	0/168	86/168	167/168	0/168	86/168	167/168
Reference POD	0.00 (0.00, 0.02)	0.51 (0.43, 0.59)	0.99 (0.97, 1.00)	0.00 (0.00, 0.02)	0.51 (0.43, 0.59)	0.99 (0.97, 1.00)
s_r	0.00 (0.00, 0.15)	0.51 (0.46, 0.52)	0.08 (0.07, 0.15)	0.00 (0.00, 0.15)	0.51 (0.46, 0.52)	0.08 (0.07, 0.15)
s_L	0.00 (0.00, 0.15)	0.00 (0.00, 0.12)	0.00 (0.00, 0.03)	0.00 (0.00, 0.15)	0.00 (0.00, 0.12)	0.00 (0.00, 0.03)
s_R	0.00 (0.00, 0.21)	0.51 (0.47, 0.52)	0.08 (0.07, 0.09)	0.00 (0.00, 0.21)	0.51 (0.47, 0.52)	0.08 (0.07, 0.09)
P-value	1.0000	0.9695	0.4418	1.0000	0.9695	0.4418
dLPOD (candidate vs reference)[f]	0.00 (–0.02, 0.02)	–0.02 (–0.13, 0.09)	0.01 (–0.02, 0.03)	0.01 (–0.02, 0.03)	–0.02 (–0.13, 0.09)	0.01 (–0.02, 0.03)
dLPOD (candidate presumptive vs candidate confirmed)[f]	0.01 (–0.01, 0.04)	0.01 (–0.10, 0.12)	0.00 (–0.02, 0.02)	0.01 (–0.02, 0.04)	0.01 (–0.10, 0.12)	0.00 (–0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P*-value = Homogeneity test of laboratory PODs.

[f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods.

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To reduce the risks associated with environmental contamination, follow current industry standards and local regulations for disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information. For questions about specific applications or procedures, visit www.3M.com/foodsafety or contact your local 3M representative or distributor. Review the policies recommend by the Centers for Disease Control and Prevention on dealing with pathogens (http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf).

A. Principle

The 3M Petrifilm SALX System is a chromogenic culture medium system that is intended for the rapid and specific detection and biochemical confirmation of *Salmonella* spp. from food and food process environmental samples. After enrichment in prewarmed 3M *Salmonella* Enrichment Base with 3M *Salmonella* Enrichment Supplement, the 3M Petrifilm SALX System provides presumptive positive results in as little as 40 h from low microbial background foods ($<10^4$ CFU/g) and 48 h from high microbial foods ($\geq 10^4$ CFU/g). The 3M Petrifilm SALX System does not specifically differentiate some lactose-positive *Salmonella* species (primarily *S. arizonae* and *S. diarizonae*) from other lactose-positive organisms. Refer to the 3M Petrifilm *Salmonella* Express System Instructions for Use for additional information.

B. Apparatus and Reagents

(**a**) *3M Petrifilm Salmonella Express Plate.*—Twenty-five plates/pouch (3M Food Safety, St. Paul, MN, USA).

(**b**) *3M Petrifilm Salmonella Express Confirmation Disk.*—Five disks/pouch (3M Food Safety).

(**c**) *3M Salmonella Enrichment Base.*—500 g or 2.5 kg/bottle (3M Food Safety).

(**d**) *3M Salmonella Enrichment Supplement.*—1 g/vial (3M Food Safety).

(**e**) *3M Petrifilm Flat Spreader.*—Two spreaders/box (3M Food Safety).

Table 2014.01B. Summary of results for detection of *Salmonella* in dry dog food (375 g)

Method[a]	3M Petrifilm *Salmonella* Express System with alternative confirmation			3M Petrifilm *Salmonella* Express System with traditional confirmation		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. of samples analyzed	0/144	82/144	142/144	0/144	82/144	142/144
Candidate presumptive POD (CP)	0.00 (0.00, 0.03)	0.57 (0.48, 0.66)	0.99 (0.95, 1.00)	0.00 (0.00, 0.03)	0.57 (0.48, 0.66)	0.99 (0.95, 1.00)
s_r[b]	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.12 (0.11, 0.16)	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.12 (0.11, 0.16)
s_L[c]	0.00 (0.00, 0.16)	0.08 (0.00, 0.24)	0.00 (0.00, 0.04)	0.00 (0.00, 0.16)	0.08 (0.00, 0.24)	0.00 (0.00, 0.04)
s_R[d]	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.12 (0.11, 0.13)	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.12 (0.11, 0.13)
P-value[e]	1.0000	0.2242	0.9861	1.0000	0.2242	0.9861
Candidate confirmed positive/ total No. of samples analyzed	0/144	81/144	141/144	0/144	82/144	141/144
Candidate confirmed POD (CC)	0.00 (0.00, 0.03)	0.56 (0.46, 0.66)	0.98 (0.94, 0.99)	0.00 (0.00, 0.03)	0.57 (0.48, 0.67)	0.98 (0.94, 0.99)
s_r	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.14 (0.12, 0.16)	0.00 (0.00, 0.16)	0.49 (0.43, 0.52)	0.14 (0.12, 0.16)
s_L	0.00 (0.00, 0.16)	0.10 (0.00, 0.26)	0.03 (0.00, 0.08)	0.00 (0.00, 0.16)	0.11 (0.00, 0.27)	0.03 (0.00, 0.08)
s_R	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.14 (0.13, 0.17)	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.14 (0.13, 0.17)
P-value	1.0000	0.1290	0.0976	1.0000	0.1114	0.0976
Positive reference samples/ total No. of samples analyzed	0/144	71/144	144/144	0/144	71/144	144/144
Reference POD	0.00 (0.00, 0.03)	0.49 (0.39, 0.59)	1.00 (0.97, 1.00)	0.00 (0.00, 0.03)	0.49 (0.39, 0.59)	1.00 (0.97, 1.00)
s_r	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.16)	0.10 (0.00, 0.26)	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)	0.10 (0.00, 0.26)	0.00 (0.00, 0.16)
s_R	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.00 (0.00, 0.22)	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.00 (0.00, 0.22)
P-value	1.0000	0.1550	1.0000	1.0000	0.1550	1.0000
dLPOD (C vs R)[f]	0.00 (–0.03, 0.03)	0.07 (–0.07, 0.21)	–0.02 (–0.06, 0.01)	0.00 (–0.03, 0.03)	0.08 (–0.07, 0.22)	–0.02 (–0.06, 0.01)
dLPOD (CP vs CC)[f]	0.00 (–0.03, 0.03)	0.01 (–0.18, 0.22)	0.01 (–0.03, 0.05)	0.00 (–0.03, 0.03)	0.00 (–0.14, 0.14)	0.01 (–0.03, 0.05)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P*-value = Homogeneity test of laboratory PODs.

[f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods.

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Table 2014.01C. Sample matrix and enrichment scheme[a]

Sample matrix	Sample size, g	Enrichment broth volume, mL	Enrichment time, h	Secondary enrichment time, h
Raw ground beef (80% lean)	25	225	18–24	8–24
Raw ground chicken	25	225	18–24	8–24
Raw ground pork	25	225	18–24	8–24
Frozen uncooked shrimp	25	225	18–24	8–24
Fresh bunched spinach	25	225	18–24	24
Stainless steel; environmental sponges	1 Sponge (4 × 4 in.)	225	18–24	
Pasteurized liquid whole egg	100	900	18–24	
Cooked breaded chicken	325	2925	18–24	
Dry dog food	375	3375	18–24	

[a] AOAC RI Certificate No. 061301.

(**f**) *3M Rappaport-Vassiliadis R10 (R-V R10) Broth.*—500 g/bottle (3M Food Safety).

(**g**) *Sterile diluents.*—Butterfield's Phosphate Diluent, distilled water, or reverse osmosis water.

(**h**) *Sterile 10 µL inoculation loop.*

(**i**) *Pipet.*—Capable of dispensing 2 mL.

(**j**) *Pipettor.*—Capable of dispensing 100 µL.

(**k**) *Sterile pipet tips.*—Capable of 100 µL.

(**l**) *Filter stomacher bags.*—Seward Laboratory Systems Inc. (Bohemia, NY, USA), or equivalent.

(**m**) *Stomacher.*—Seward Laboratory Systems Inc., or equivalent.

(**n**) *Permanent ultra-fine tipped marker.*—For circling presumptive positive colonies on the 3M Petrifilm *Salmonella* Express Plate.

(**o**) *Incubators.*—Capable of maintaining 41.5 ± 1°C.

(**p**) *Freezer.*—Capable of maintaining –10 to –20°C, for storing opened 3M Petrifilm *Salmonella* Express Plate pouches, hydrated 3M Petrifilm SALX Plates, and 3M Petrifilm SALX Plates after incubation.

(**q**) *Refrigerator.*—Capable of maintaining 2–8°C for storing unopened 3M Petrifilm SALX Plates and 3M Petrifilm SALX Confirmation Disk.

C. General Instructions

(**a**) Store 3M Petrifilm SALX Plates and 3M Petrifilm SALX Confirmation Disks at 2–8°C. After opening the 3M Petrifilm SALX Plate pouches, seal the pouch and store at ambient temperature, less than 60% relative humidity (RH). Hydrated 3M Petrifilm SALX Plates can be stored up to 7 days at 2–8°C. Post-incubation 3M Petrifilm SALX Plates can be stored at –10 to –20°C for up to 3 days. Hydrate the 3M Petrifilm SALX Plates with 2.0 ± 0.1 mL sterile diluent. Do not allow the top film to close before dispensing the entire 2.0 mL volume. Gently roll down the top film onto the diluent to prevent trapping air bubbles. Place the 3M Petrifilm Flat Spreader on the center of the plate. Press gently on the center of the spreader to distribute the diluent evenly. Spread the diluent over the entire 3M Petrifilm SALX Plate. Remove the spreader and leave the 3M Petrifilm SALX Plate undisturbed for 1 min. Prior to use, place the plates on a flat surface for 1 h at room temperature (20–25°C/<60% RH) and protected from light to allow the gel to form. Hydrated plates can be stored at room temperature (20–25°C/<60% RH) protected from light for up to 8 h before use.

(**b**) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

(**c**) After use, the enrichment medium and the 3M Petrifilm SALX Plates and 3M Petrifilm SALX Confirmation Disks can potentially contain pathogenic materials. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal.

D. Sample Enrichment

(*1*) Prewarm 3M *Salmonella* Enrichment Base with 3M *Salmonella* Enrichment Supplement (50 mg/L) to 41.5 ± 1°C.

(*2*) Aseptically combine the enrichment medium and sample following Table **2014.01C**. For all meat and highly particulate samples, the use of filter bags is recommended. Homogenize thoroughly for 2 min and incubate at 41.5 ± 1°C for 18–24 h.

(*a*) *Foods with high microbial backgrounds (≥10^4 CFU/g).*—Transfer 0.1 mL of the primary enrichment into 10.0 mL R-V R10 broth. Incubate for 8–24 h at 41.5 ± 1°C.

(*b*) *Foods with low microbial backgrounds (<10^4 CFU/g).*—Proceed to 3M Petrifilm SALX Plate preparation as described in **E**.

E. Preparation of the 3M Petrifilm Salmonella Express Plates

(*1*) Place the 3M Petrifilm SALX Plate on a flat, level surface.

(*2*) Use prescribed diluents to hydrate the 3M Petrifilm SALX Plates: Butterfield's Phosphate Diluent, distilled water, or reverse osmosis water.

(*3*) Lift the top film and with the pipet perpendicular dispense 2.0±0.1 mL sterile diluent onto the center of bottom film. Do not close the top film before dispensing the entire 2.0 mL volume.

(*4*) Gently roll down the top film onto the diluent to prevent trapping air bubbles.

(*5*) Place the 3M Petrifilm Flat Spreader on the center of the plate. Press gently on the center of the spreader to distribute the diluent evenly. Spread the diluent over the entire 3M Petrifilm SALX Plate growth area before the gel is formed. Do not slide the spreader across the film.

(*6*) Remove the spreader and leave the 3M Petrifilm SALX Plate undisturbed for at least 1 min.

(*7*) Place 3M Petrifilm SALX Plate on a flat surface for at least 1 h at room temperature (20–25°C/<60% RH), protected from light to allow the gel to form prior to use. Hydrated 3M Petrifilm SALX

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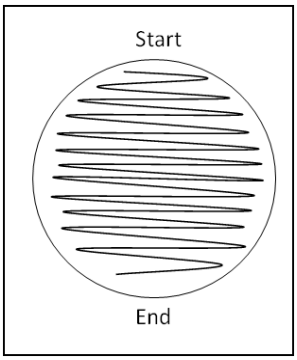

Figure 2014.01. Streaking pattern on the 3M Petrifilm SALX Plate.

Plates can be stored at room temperature (20–25°C/<60% RH) for up to 8 h before use if protected from light.

(*8*) If hydrated plates are not used within 8 h, store in a sealed plastic bag, protected from light, and store at –20 to –10°C for up to 5 days.

F. 3M Petrifilm Salmonella Express Plate Inoculation

(*1*) Remove the enrichment medium from the incubator and agitate contents by hand.

(*2*) Use a sterile 10 µL loop (3 mm diameter) to withdraw each sample. Use a smooth loop (one that does not have jagged edges and is not distorted) to prevent the gel surface from breaking.

(*3*) Open the 3M Petrifilm SALX Plate and streak onto the gel. Perform a single streak to obtain isolated colonies (Figure **2014.01**).

(*4*) Roll down the top film to close the 3M Petrifilm SALX Plate.

(*5*) Using a gloved hand (while practicing GLP to avoid cross-contamination and/or direct contact with the plate), gently apply a sweeping motion with even pressure onto the top film to remove any air bubbles in the inoculation area.

(*6*) Streak each enriched test portion onto a 3M Petrifilm SALX Plate and incubate at 41.5±1°C for 24±2 h in a horizontal position with the colored side up in stacks of no more than 20 plates.

G. Confirmation of 3M Petrifilm Salmonella Express Plates

(*1*) Using a permanent ultra-fine tip marker, circle at least five presumptive positive colonies (red to brown colonies with a yellow zone or associated gas bubble, or both) on the plate top film (*see* Table **2014.01D**).

Table 2014.01D. Interpretation for presumptive positive *Salmonella* species

Colony color			Colony metabolism		
Red	Dark red	Brown	Yellow zone	Gas bubble	Result
√			√		Presumptive +
√				√	Presumptive +
√			√	√	Presumptive +
	√		√		Presumptive +
	√			√	Presumptive +
	√		√	√	Presumptive +
		√	√		Presumptive +
		√		√	Presumptive +
		√	√	√	Presumptive +

(*2*) Lift the top film of the 3M Petrifilm SALX Plate and insert the 3M Petrifilm SALX Confirmation Disk by rolling it onto the gel to avoid entrapping air bubbles. Close the 3M Petrifilm SALX Plate. Using a gloved hand, gently apply a sweeping motion with even pressure onto the top film to remove any air bubbles in the inoculation area and ensure good contact between the gel and the 3M Petrifilm SALX Confirmation Disk.

(*3*) Incubate the 3M Petrifilm SALX System (plate and disk) at 41.5±1°C for 4–5 h in a horizontal position, right side up, in stacks of no more than 20 plates.

(*4*) Observe circled colonies for color change. Red/brown to green blue, blue, dark blue, or black confirms the colony as *Salmonella* spp. No color change indicates the colony is negative. If presumptive positive *Salmonella* colonies are not present, then report the results as *Salmonella* not detected in the matrix.

(*5*) Transfer typical colonies from 3M Petrifilm SALX Plate to TSI/LIA slants. Incubate 35±1°C for 24±2 h.

(*6*) Confirm a minimum of one typical colony per test portion with biochemical/serological procedures prescribed by the current versions of the USDA/FSIS-MLG or FDA/BAM reference methods.

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Candidates for 2016 Method of the Year

FOOD BIOLOGICAL CONTAMINANTS

Evaluation of the 3M™ Petrifilm™ *Salmonella* Express System for the Detection of *Salmonella* Species in Selected Foods: Collaborative Study

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The 3M™ Petrifilm™ *Salmonella* Express (SALX) System is a simple, ready-to-use chromogenic culture medium system for the rapid qualitative detection and biochemical confirmation of *Salmonella* spp. in food and food process environmental samples. The 3M Petrifilm SALX System was compared using an unpaired study design in a multilaboratory collaborative study to the U.S. Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) *Microbiology Laboratory Guidebook* (MLG) 4.07 (2013) *Isolation and Identification of Salmonella from Meat, Poultry, Pasteurized Egg and Catfish Products and Carcass and Environmental Sponges* for raw ground beef and the U.S. Food and Drug Administration *Bacteriological Analytical Manual* (FDA/BAM) Chapter 5, *Salmonella* (2011) reference method for dry dog food following the current AOAC validation guidelines. For this study, a total of 17 laboratories located throughout the continental United States evaluated 1872 test portions. For the 3M Petrifilm SALX System, raw ground beef was analyzed using 25 g test portions, and dry dog food was analyzed using 375 g test portions. For the reference methods, 25 g test portions of each matrix were analyzed. The two matrices were artificially contaminated with *Salmonella* at three inoculation levels: an uninoculated control level (0 CFU/test portion), a low inoculum level (0.2–2 CFU/test portion), and a high inoculum level (2–5 CFU/test portion). Each inoculation level was statistically analyzed using the probability of detection statistical model. For the raw ground beef and dry dog food test portions, no significant differences at the 95% confidence interval were observed in the number of positive samples detected by the 3M Petrifilm SALX System versus either the USDA/FSIS-MLG or FDA/BAM methods.

In the last quarter century, significant efforts have been made to reduce the occurrence of *Salmonella* in food products, yet *Salmonella* spp. continues to be the most frequently reported cause of foodborne illness in the United States (1). Over 2500 different serotypes of *Salmonella* spp. have been isolated from a wide range of food products including raw meats and poultry, shell eggs, chocolate, fresh fruit and vegetables, and low-moisture ingredients such as spices and peanut butter (2). This broad range of implicated products further illustrates why testing for and confirming the presence of *Salmonella* as rapidly as possible is so critical to food safety. The 3M™ Petrifilm™ *Salmonella* Express (SALX) System, a chromogenic culture medium system, uses a cold-water-soluble gelling agent to selectively differentiate *Salmonella* from background flora in enriched food and food process environmental samples.

The 3M Petrifilm SALX System allows for the rapid and specific detection and biochemical confirmation of *Salmonella* species from food and environmental samples. Following enrichment in 3M™ *Salmonella* Enrichment Base containing 3M™ *Salmonella* Enrichment Supplement, the 3M Petrifilm SALX System can provide presumptive results in as little as 40 h from low microbial background foods ($<10^4$ CFU/g) and 48 h from high-microbial background foods ($\geq 10^4$ CFU/g). Confirmation of multiple presumptive *Salmonella* colonies at once is accomplished using the 3M™ Petrifilm™ *Salmonella* Express (SALX) Confirmation Disk which uses biochemical enzymes to facilitate the reaction. The method developer studies demonstrated that the 3M Petrifilm SALX System did not specifically differentiate some lactose-positive *Salmonella* species (primarily *S. arizonae* and *S. diarizonae*) from other lactose-positive organisms.

Prior to the collaborative study, the 3M Petrifilm SALX System was validated according to AOAC Validation Guidelines (3) in a harmonized AOAC *Performance Tested Method*SM (PTM) study. The objective of the PTM study

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The Expert Review Panel for Microbiology Methods for Food and Environmental Surfaces invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author or methodfeedback@aoac.org.
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An appendix is available on the *J. AOAC Int.* website, http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
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was to demonstrate that the 3M Petrifilm SALX System detects *Salmonella* spp. in selected foods as claimed by the manufacturer. For the 3M Petrifilm SALX System evaluation, nine matrices were evaluated: raw ground beef (25 g), raw ground chicken (25 g), pasteurized liquid whole egg (100 g), raw ground pork (25 g), cooked chicken nuggets (325 g), frozen uncooked shrimp (25 g), fresh bunched spinach (25 g), dry dog food (375 g), and stainless steel.

All other PTM parameters (inclusivity, exclusivity, ruggedness, stability, and lot-to-lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method was awarded PTM certification No. 061301 on June 5, 2013.

The aim of this collaborative study was to compare the 3M Petrifilm SALX System to the U.S. Department of Agriculture (USDA) Food Safety Inspection Service (FSIS)/*Microbiology Laboratory Guidebook* (MLG) 4.07 (4) for raw ground beef, and the U.S. Food and Drug Administration (FDA) *Bacteriological Analytical Manual* (BAM) Chapter 5 (5) method for dry dog food.

Collaborative Study

Study Design

For this collaborative study, two matrices, raw ground beef (80% lean) and dry dog food, were evaluated. The matrices were obtained from local retailers and screened for the presence of *Salmonella* spp. by either the MLG or BAM reference methods. The raw ground beef was artificially contaminated with *Salmonella* Ohio Sequence Types 81 (University of Pennsylvania Culture Collection) and the dry dog food with *Salmonella* Poona National Collection of Type Cultures (NCTC) 4840. There were three inoculation levels for each matrix: a high inoculation level of approximately 2–5 CFU/test portion, a low inoculation level of approximately 0.2–2 CFU/test portion, and an uninoculated control level at 0 CFU/test portion. Twelve replicate samples from each of the three inoculation levels of product were analyzed by both the candidate and reference method. Two sets of unpaired samples (72 total) were sent to each laboratory for analysis by the 3M Petrifilm SALX System and either the MLG (raw ground beef) or BAM (dry dog food) reference method due to differences in enrichment protocols. For both matrices, collaborators were sent an additional 60 g test portion and instructed to conduct a total aerobic plate count (APC) using 3M™ Petrifilm™ Aerobic Count Plate (AOAC *Official Method* **990.12**) on the day samples were received. Foods with an APC count of greater than or equal to 1.0×10^4 CFU/g were categorized as high microbial load foods, and those foods lower than 1.0×10^4 CFU/g were categorized as low microbial load. A detailed collaborative study packet outlining all necessary information related to the study including media preparation, method-specific test portion preparation, and documentation of results was sent to each collaborating laboratory prior to the initiation of the study.

Preparation of Inocula and Test Portions

The *Salmonella* cultures used in this evaluation were propagated in 10 mL Brain Heart Infusion (BHI) broth from a frozen stock culture stored at –70°C at Q Laboratories, Inc. The broth was incubated for 18–24 h at 35 ± 1°C. For both matrices, a bulk lot of each matrix was inoculated with a liquid inoculum and mixed thoroughly by hand-kneading to ensure an even distribution of microorganisms. Appropriate dilutions of the cultures were prepared based on previously established growth curves for both low and high inoculation levels, resulting in fractional positive outcomes for at least one level. For the dry pet food, prior to inoculation, the inoculum was heat-stressed in a 50°C water bath for 10 min to obtain a percent injury of 50–80% (as determined by plating onto selective xylose deoxycholate agar and nonselective tryptic soy agar). The degree of injury was estimated as:

$$(1 - \frac{n_{select}}{n_{nonselect}})x100$$

where n_{select} = number of colonies on selective agar, and $n_{nonselect}$ = number of colonies on nonselective agar. The raw ground beef was inoculated on the day of shipment so that the organism had equilibrated within the matrix for 96 h before testing was initiated. Dry dog food was inoculated and held at room temperature (24 ± 2°C) so that the organism would have equilibrated for a minimum of 2 weeks prior to initiation of testing. The shipment and hold times of the inoculated test material were verified as a QC measure prior to study initiation. For the evaluation of the raw ground beef, the bulk lot of inoculated test material was divided into 30 g portions for shipment to the collaborators. For the evaluation of the dry dog food, 25 g of inoculated test product was mixed with 350 g of uninoculated test product for shipment to the collaborators for the analysis by the 3M Petrifilm SALX System. For analysis by the reference methods, collaborators received 30 g portions. Validation criterion were satisfied when inoculated test portions produced fractional recovery of the spiked organism, defined as either the reference or candidate method yielding 25–75% positive results.

To determine the level of *Salmonella* spp. in the matrices, a five-tube most probable number (MPN) was conducted at Q Laboratories, Inc. on the day of initiation of analysis using the BAM Chapter 5 reference method for dry dog food or the MLG 4.07 reference method for raw ground beef. From both the high and low inoculated levels, five 100 g test portions, the reference method test portions from the collaborating laboratories, and five 10 g test portions were analyzed following the appropriate reference method. The MPN and 95% confidence intervals were calculated from the high, medium, and low levels using the Least Cost Formulations (LCF) MPN Calculator , Version 1.6, provided by AOAC (www.lcfltd.com/customer/LCFMPNCalculator.exe; 6). Confirmation of the samples was conducted according to the appropriate reference method, dependent on the matrix.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded three digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by International Air Transport Association. Raw ground beef samples were packed with cold packs to target a temperature of <7°C during shipment. Upon receipt, samples were held by the collaborating laboratory at refrigerated temperature (3–5°C) until the following Monday when analysis was initiated. Dry dog

food samples were packed and shipped at ambient temperature. Upon receipt, samples were held by the collaborating laboratory at room temperature (24 ± 2°C). In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as "temperature control." Participants were instructed to record the temperature of this portion upon receipt of the shipment, document results on the Sample Receipt Confirmation form provided, and fax to the Study Director. For both matrices, several shipments were delayed from getting to the testing facilities on time due to inclement weather. Upon receiving their packages on either Saturday or Monday, the testing laboratories were instructed to document the temperature of the samples and to continue testing. No laboratories were solely excluded from testing due to the delay in package receipt.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method specified for each matrix. For both matrices, each collaborator received 72 test portions of each food product (12 high, 12 low, and 12 controls for each method). For the analysis of the raw ground beef test portions by the 3M Petrifilm SALX System, a 25 g portion was enriched with 225 mL of prewarmed (41.5±1°C) 3M *Salmonella* Enrichment Base containing 3M *Salmonella* Enrichment Supplement (50 mg/L), homogenized for 2 min, and incubated for 18–24 h at 41.5±1°C. For the dry dog food test portions analyzed by the 3M Petrifilm SALX System, a 375 g portion was enriched with 3375 mL prewarmed (41.5±1°C) 3M *Salmonella* Enrichment Base containing 3M *Salmonella* Enrichment Supplement (50 mg/L), homogenized for 2 min, and incubated for 18–24 h at 41.5±1°C.

Following enrichment of raw ground beef samples, the enrichment protocol for high microbial load foods was followed where a 0.1 mL aliquot of each test portion was transferred into 10.0 mL Rappaport-Vassiliadis R10 (R-V R10) broth and incubated for 8–24 h at 41.5±1°C. After incubation, a loopful of the secondary enrichment was streaked directly onto hydrated 3M Petrifilm SALX Plates and incubated for 24±2 h at 41.5±1°C. For dry dog food samples, the enrichment protocol for low microbial load foods was followed where a loopful of the primary enrichment was streaked directly onto hydrated 3M Petrifilm SALX Plates and incubated for 24±2 h at 41.5±1°C. For both matrices, the 3M Petrifilm SALX Plates were examined for typical colonies (red to brown colony with a yellow zone or associated gas bubble, or both).

Typical colonies were circled on the plate top film using a fine tip permanent black marker. The top of the 3M Petrifilm SALX Plate was lifted and a 3M Petrifilm SALX Confirmation Disk was placed onto the gel. The film was lowered and air bubbles were removed using a sweeping motion. The plates were incubated for 4–5 h at 41.5±1°C. After incubation, the circled colonies were observed for color change: red/brown to green blue, blue, dark blue, or black. Typical colonies were transferred to triple sugar iron/lysine iron agar (TSI/LIA) slants and confirmed following the standard reference methods. Additionally, for each matrix analyzed by the 3M Petrifilm SALX System, aliquots of the primary enrichment were transferred to the secondary enrichments and confirmed following procedures outlined in the MLG or BAM.

Both test portion sizes analyzed by the 3M Petrifilm SALX System were compared to samples (25 g) analyzed using either the MLG or BAM reference method in an unpaired study design. All positive test portions were biochemically confirmed by the API 20E biochemical test, AOAC *Official Method* **978.24** or by the VITEK 2 GN identification test, AOAC *Official Method* **2011.17**. The biochemical method was determined by each individual participating laboratory based on their current method used for confirmation of routine samples. Serological testing, Group Poly O A-I & Vi and Poly H latex agglutination, was also performed.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and the 3M Petrifilm SALX System on the data sheets provided in the collaborative study outline or the electronic spreadsheet created as a result of multiple requests for electronic data entry. The data sheets were submitted to the Study Director at the end of each week of testing for analysis. The results of each test portion for each sample were compiled by the Study Director and the qualitative 3M Petrifilm SALX System results were compared to the reference methods for statistical analysis. Data for each test portion size were analyzed using the probability of detection (POD) statistical model (3, 7). A confidence interval of a dLPOD (difference between the POD of the reference and candidate method) not containing the point zero would indicate a statistically significant difference between the 3M Petrifilm SALX System and the MLG or BAM reference methods at the 5% probability level (8). In addition to calculating the POD for each inoculation level, the repeatability standard deviation, among-laboratory standard deviation, reproducibility standard deviation, and a *P*-value for homogeneity were calculated. For the collaborative study, the 3M Petrifilm SALX System produced 479 presumptive positive results with 475 confirming positive by the traditional confirmation and 473 confirming positive by the alternative confirmation. There were 468 confirmed positives by the reference method.

AOAC Official Method 2014.01
Salmonella in Selected Foods
3M™ Petrifilm™ *Salmonella* Express System
First Action 2014

[Applicable to detection of *Salmonella* spp. in raw ground beef (25 g), raw ground chicken (25 g), pasteurized liquid whole egg (100 g), raw ground pork (25 g), cooked chicken nuggets (325 g), frozen uncooked shrimp (25 g), fresh bunched spinach (25 g), dry dog food (375 g), and stainless steel. Not applicable to some lactose-positive *Salmonella* species.]

See Tables **2014.01A** and **B** for results of the interlaboratory study supporting acceptance of the method. *See* Appendix available on the *J. AOAC Int.* website for detailed tables of results of the collaborative study.

Caution: Do not use the 3M Petrifilm SALX System method in the diagnosis of conditions in humans or animals. To reduce the risks associated with exposure to chemicals and biohazards, perform pathogen testing in a properly equipped laboratory under the control of trained personnel. Always follow

Table 2014.01A. Summary of results for detection of *Salmonella* in raw ground beef (25 g)

Method[a]	3M Petrifilm *Salmonella* Express System with alternative confirmation			3M Petrifilm *Salmonella* Express System with traditional confirmation		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. of samples analyzed	2/168	85/168	168/168	2/168	85/168	168/168
Candidate presumptive POD (CP)	0.01 (0.00, 0.04)	0.51 (0.43, 0.58)	1.00 (0.98, 1.00)	0.01 (0.00, 0.04)	0.51 (0.43, 0.58)	1.00 (0.98, 1.00)
s_r[b]	0.11 (0.10, 0.15)	0.51 (0.46, 0.52)	0.00 (0.00, 0.15)	0.11 (0.10, 0.15)	0.51 (0.46, 0.52)	0.00 (0.00, 0.15)
s_L[c]	0.00 (0.00, 0.04)	0.00 (0.00, 0.13)	0.00 (0.00, 0.15)	0.00 (0.00, 0.04)	0.00 (0.00, 0.13)	0.00 (0.00, 0.15)
s_R[d]	0.11 (0.10, 0.12)	0.51 (0.47, 0.52)	0.00 (0.00, 0.21)	0.11 (0.10, 0.12)	0.51 (0.47, 0.52)	0.00 (0.00, 0.21)
P-value[e]	0.5158	0.9341	1.0000	0.5158	0.9341	1.0000
Candidate confirmed positive/ total No. of samples analyzed	0/168	83/168	168/168	1/168	83/168	168/168
Candidate confirmed POD (CC)	0.00 (0.00, 0.02)	0.49 (0.42, 0.57)	1.00 (0.98, 1.00)	0.01 (0.00, 0.03)	0.49 (0.42, 0.57)	1.00 (0.98, 1.00)
s_r	0.00 (0.00, 0.15)	0.51 (0.46, 0.52)	0.00 (0.00, 0.15)	0.08 (0.07, 0.15)	0.51 (0.46, 0.52)	0.00 (0.00, 0.15)
s_L	0.00 (0.00, 0.15)	0.00 (0.00, 0.11)	0.00 (0.00, 0.15)	0.00 (0.00, 0.03)	0.00 (0.00, 0.11)	0.00 (0.00, 0.15)
s_R	0.00 (0.00, 0.21)	0.51 (0.47, 0.52)	0.00 (0.00, 0.21)	0.08 (0.07, 0.09)	0.51 (0.47, 0.52)	0.00 (0.00, 0.21)
P-value	1.0000	0.9757	1.0000	0.4418	0.9757	1.0000
Positive reference samples/ total No. of samples analyzed	0/168	86/168	167/168	0/168	86/168	167/168
Reference POD	0.00 (0.00, 0.02)	0.51 (0.43, 0.59)	0.99 (0.97, 1.00)	0.00 (0.00, 0.02)	0.51 (0.43, 0.59)	0.99 (0.97, 1.00)
s_r	0.00 (0.00, 0.15)	0.51 (0.46, 0.52)	0.08 (0.07, 0.15)	0.00 (0.00, 0.15)	0.51 (0.46, 0.52)	0.08 (0.07, 0.15)
s_L	0.00 (0.00, 0.15)	0.00 (0.00, 0.12)	0.00 (0.00, 0.03)	0.00 (0.00, 0.15)	0.00 (0.00, 0.12)	0.00 (0.00, 0.03)
s_R	0.00 (0.00, 0.21)	0.51 (0.47, 0.52)	0.08 (0.07, 0.09)	0.00 (0.00, 0.21)	0.51 (0.47, 0.52)	0.08 (0.07, 0.09)
P-value	1.0000	0.9695	0.4418	1.0000	0.9695	0.4418
dLPOD (candidate vs reference)[f]	0.00 (–0.02, 0.02)	–0.02 (–0.13, 0.09)	0.01 (–0.02, 0.03)	0.01 (–0.02, 0.03)	–0.02 (–0.13, 0.09)	0.01 (–0.02, 0.03)
dLPOD (candidate presumptive vs candidate confirmed)[f]	0.01 (–0.01, 0.04)	0.01 (–0.10, 0.12)	0.00 (–0.02, 0.02)	0.01 (–0.02, 0.04)	0.01 (–0.10, 0.12)	0.00 (–0.02, 0.02)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P*-value = Homogeneity test of laboratory PODs.

[f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods.

standard good laboratory safety practices (GLP), including proper containment procedures, and wearing appropriate protective apparel and eye protection while handling testing materials and test samples. Avoid direct contact with the contents of the enrichment medium and inoculated plates. Dispose of enrichment media and inoculated plates according to all applicable government regulatory regulations and applicable laboratory procedures. Wear appropriate protective apparel while handling the 3M Petrifilm SALX Plate as some of the components may be considered allergenic and irritants to some individuals.

To reduce the risks associated with environmental contamination, follow current industry standards and local regulations for disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information. For questions about specific applications or procedures, visit www.3M.com/foodsafety or contact your local 3M representative or distributor. Review the policies recommend by the Centers for Disease Control and Prevention on dealing with pathogens (http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf).

A. Principle

The 3M Petrifilm SALX System is a chromogenic culture medium system that is intended for the rapid and specific detection and biochemical confirmation of *Salmonella* spp. from food and food process environmental samples. After enrichment in prewarmed 3M *Salmonella* Enrichment Base with 3M *Salmonella* Enrichment Supplement, the 3M Petrifilm SALX System provides presumptive positive results in as little as 40 h from low microbial background foods ($<10^4$ CFU/g) and 48 h from high microbial foods ($\geq 10^4$ CFU/g). The 3M

Table 2014.01B. Summary of results for detection of *Salmonella* in dry dog food (375 g)

Method[a]	3M Petrifilm *Salmonella* Express System with alternative confirmation			3M Petrifilm *Salmonella* Express System with traditional confirmation		
Inoculation level	Uninoculated	Low	High	Uninoculated	Low	High
Candidate presumptive positive/ total No. of samples analyzed	0/144	82/144	142/144	0/144	82/144	142/144
Candidate presumptive POD (CP)	0.00 (0.00, 0.03)	0.57 (0.48, 0.66)	0.99 (0.95, 1.00)	0.00 (0.00, 0.03)	0.57 (0.48, 0.66)	0.99 (0.95, 1.00)
s_r[b]	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.12 (0.11, 0.16)	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.12 (0.11, 0.16)
s_L[c]	0.00 (0.00, 0.16)	0.08 (0.00, 0.24)	0.00 (0.00, 0.04)	0.00 (0.00, 0.16)	0.08 (0.00, 0.24)	0.00 (0.00, 0.04)
s_R[d]	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.12 (0.11, 0.13)	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.12 (0.11, 0.13)
P-value[e]	1.0000	0.2242	0.9861	1.0000	0.2242	0.9861
Candidate confirmed positive/ total No. of samples analyzed	0/144	81/144	141/144	0/144	82/144	141/144
Candidate confirmed POD (CC)	0.00 (0.00, 0.03)	0.56 (0.46, 0.66)	0.98 (0.94, 0.99)	0.00 (0.00, 0.03)	0.57 (0.48, 0.67)	0.98 (0.94, 0.99)
s_r	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.14 (0.12, 0.16)	0.00 (0.00, 0.16)	0.49 (0.43, 0.52)	0.14 (0.12, 0.16)
s_L	0.00 (0.00, 0.16)	0.10 (0.00, 0.26)	0.03 (0.00, 0.08)	0.00 (0.00, 0.16)	0.11 (0.00, 0.27)	0.03 (0.00, 0.08)
s_R	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.14 (0.13, 0.17)	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.14 (0.13, 0.17)
P-value	1.0000	0.1290	0.0976	1.0000	0.1114	0.0976
Positive reference samples/ total No. of samples analyzed	0/144	71/144	144/144	0/144	71/144	144/144
Reference POD	0.00 (0.00, 0.03)	0.49 (0.39, 0.59)	1.00 (0.97, 1.00)	0.00 (0.00, 0.03)	0.49 (0.39, 0.59)	1.00 (0.97, 1.00)
s_r	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)	0.49 (0.44, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.16)	0.10 (0.00, 0.26)	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)	0.10 (0.00, 0.26)	0.00 (0.00, 0.16)
s_R	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.00 (0.00, 0.22)	0.00 (0.00, 0.22)	0.50 (0.45, 0.52)	0.00 (0.00, 0.22)
P-value	1.0000	0.1550	1.0000	1.0000	0.1550	1.0000
dLPOD (C vs R)[f]	0.00 (–0.03, 0.03)	0.07 (–0.07, 0.21)	–0.02 (–0.06, 0.01)	0.00 (–0.03, 0.03)	0.08 (–0.07, 0.22)	–0.02 (–0.06, 0.01)
dLPOD (CP vs CC)[f]	0.00 (–0.03, 0.03)	0.01 (–0.18, 0.22)	0.01 (–0.03, 0.05)	0.00 (–0.03, 0.03)	0.00 (–0.14, 0.14)	0.01 (–0.03, 0.05)

[a] Results include 95% confidence intervals.

[b] Repeatability standard deviation.

[c] Among-laboratory standard deviation.

[d] Reproducibility standard deviation.

[e] *P*-value = Homogeneity test of laboratory PODs.

[f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods.

Petrifilm SALX System does not specifically differentiate some lactose-positive *Salmonella* species (primarily *S. arizonae* and *S. diarizonae*) from other lactose-positive organisms. Refer to the 3M Petrifilm *Salmonella* Express System Instructions for Use for additional information.

B. Apparatus and Reagents

(**a**) *3M Petrifilm Salmonella Express Plate.*—Twenty-five plates/pouch (3M Food Safety, St. Paul, MN).

(**b**) *3M Petrifilm Salmonella Express Confirmation Disk.*—Five disks/pouch (3M Food Safety).

(**c**) *3M Salmonella Enrichment Base.*—500 g or 2.5 kg/bottle (3M Food Safety).

(**d**) *3M Salmonella Enrichment Supplement.*—1 g/vial (3M Food Safety).

(**e**) *3M™ Petrifilm™ Flat Spreader.*—Two spreaders/box (3M Food Safety).

(**f**) *3M Rappaport-Vassiliadis R10 (R-VR10) Broth.*—500 g/bottle (3M Food Safety).

(**g**) *Sterile diluents.*—Butterfield's Phosphate Diluent, distilled water, or reverse osmosis water.

(**h**) *Sterile 10 µL inoculation loop.*

(**i**) *Pipet.*—Capable of dispensing 2 mL.

(**j**) *Pipettor.*—Capable of dispensing 100 µL.

(**k**) *Sterile pipet tips.*—Capable of 100 µL.

(**l**) *Filter stomacher bags.*—Seward Laboratory Systems Inc., Bohemia, NY, or equivalent.

(**m**) *Stomacher.*—Seward Laboratory Systems Inc., or equivalent.

(**n**) *Permanent ultra-fine tipped marker.*—For circling presumptive positive colonies on the 3M Petrifilm *Salmonella* Express Plate.

(**o**) *Incubators.*—Capable of maintaining 41.5 ± 1°C.

(**p**) *Freezer.*—Capable of maintaining –10 to –20°C, for storing opened 3M Petrifilm *Salmonella* Express Plate pouches,

Table 2014.01C. Sample matrix and enrichment scheme[a]

Sample matrix	Sample size, g	Enrichment broth volume, mL	Enrichment time, h	Secondary enrichment time, h
Raw ground beef (80% lean)	25	225	18–24	8–24
Raw ground chicken	25	225	18–24	8–24
Raw ground pork	25	225	18–24	8–24
Frozen uncooked shrimp	25	225	18–24	8–24
Fresh bunched spinach	25	225	18–24	24
Stainless steel; environmental sponges	1 Sponge (4 × 4 in.)	225	18–24	
Pasteurized liquid whole egg	100	900	18–24	
Cooked breaded chicken	325	2925	18–24	
Dry dog food	375	3375	18–24	

[a] AOAC RI Certificate No. 061301.

hydrated 3M Petrifilm SALX Plates, and 3M Petrifilm SALX Plates after incubation.

(**q**) *Refrigerator.*—Capable of maintaining 2–8°C for storing unopened 3M Petrifilm SALX Plates and 3M Petrifilm SALX Confirmation Disk.

C. General Instructions

(**a**) Store 3M Petrifilm SALX Plates and 3M Petrifilm SALX Confirmation Disks at 2–8°C. After opening the 3M Petrifilm SALX Plate pouches, seal the pouch and store at ambient temperature, less than 60% relative humidity (RH). Hydrated 3M Petrifilm SALX Plates can be stored up to 7 days at 2–8°C. Post-incubation 3M Petrifilm SALX Plates can be stored at –10 to –20°C for up to 3 days. Hydrate the 3M Petrifilm SALX Plates with 2.0 ± 0.1 mL sterile diluent. Do not allow the top film to close before dispensing the entire 2.0 mL volume. Gently roll down the top film onto the diluent to prevent trapping air bubbles. Place the 3M Petrifilm Flat Spreader on the center of the plate. Press gently on the center of the spreader to distribute the diluent evenly. Spread the diluent over the entire 3M Petrifilm SALX Plate. Remove the spreader and leave the 3M Petrifilm SALX Plate undisturbed for 1 min. Prior to use, place the plates on a flat surface for 1 h at room temperature (20–25°C/<60% RH) and protected from light to allow the gel to form. Hydrated plates can be stored at room temperature (20–25°C/<60% RH) protected from light for up to 8 h before use.

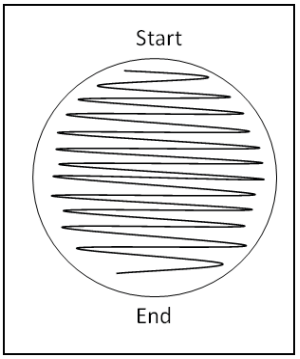

Figure 2014.01. Streaking pattern on the 3M Petrifilm SALX Plate.

(**b**) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

(**c**) After use, the enrichment medium and the 3M Petrifilm SALX Plates and 3M Petrifilm SALX Confirmation Disks can potentially contain pathogenic materials. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal.

D. Sample Enrichment

(*1*) Prewarm 3M *Salmonella* Enrichment Base with 3M *Salmonella* Enrichment Supplement (50 mg/L) to 41.5 ± 1°C.

(*2*) Aseptically combine the enrichment medium and sample following Table **2014.01C**. For all meat and highly particulate samples, the use of filter bags is recommended. Homogenize thoroughly for 2 min and incubate at 41.5 ± 1°C for 18–24h.

(*a*) *Foods with high microbial backgrounds (≥10^4 CFU/g).*—Transfer 0.1 mL of the primary enrichment into 10.0 mL R-V R10 broth. Incubate for 8–24 h at 41.5 ± 1°C.

(*b*) *Foods with low microbial backgrounds (<10^4 CFU/g).*—Proceed to 3M Petrifilm SALX Plate preparation as described in **E**.

E. Preparation of the 3M Petrifilm Salmonella Express Plates

(*1*) Place the 3M Petrifilm SALX Plate on a flat, level surface.

(*2*) Use prescribed diluents to hydrate the 3M Petrifilm SALX Plates: Butterfield's Phosphate Diluent, distilled water, or reverse osmosis water.

(*3*) Lift the top film and with the pipet perpendicular dispense 2.0±0.1 mL sterile diluent onto the center of bottom film. Do not close the top film before dispensing the entire 2.0 mL volume.

(*4*) Gently roll down the top film onto the diluent to prevent trapping air bubbles.

(*5*) Place the 3M Petrifilm Flat Spreader on the center of the plate. Press gently on the center of the spreader to distribute the diluent evenly. Spread the diluent over the entire 3M Petrifilm SALX Plate growth area before the gel is formed. Do not slide the spreader across the film.

Table 2014.01D. Interpretation for presumptive positive *Salmonella* species

Colony color			Colony metabolism		
Red	Dark red	Brown	Yellow zone	Gas bubble	Result
√			√		Presumptive +
√				√	Presumptive +
√			√	√	Presumptive +
	√		√		Presumptive +
	√			√	Presumptive +
	√		√	√	Presumptive +
		√	√		Presumptive +
		√		√	Presumptive +
		√	√	√	Presumptive +

(*6*) Remove the spreader and leave the 3M Petrifilm SALX Plate undisturbed for at least 1 min.

(*7*) Place 3M Petrifilm SALX Plate on a flat surface for at least 1 h at room temperature (20–25°C/<60% RH), protected from light to allow the gel to form prior to use. Hydrated 3M Petrifilm SALX Plates can be stored at room temperature (20–25°C/<60% RH) for up to 8 h before use if protected from light.

(*8*) If hydrated plates are not used within 8 h, store in a sealed plastic bag, protected from light, and store at –20 to –10°C for up to 5 days.

F. 3M Petrifilm Salmonella Express Plate Inoculation

(*1*) Remove the enrichment medium from the incubator and agitate contents by hand.

(*2*) Use a sterile 10 µL loop (3 mm diameter) to withdraw each sample. Use a smooth loop (one that does not have jagged edges and is not distorted) to prevent the gel surface from breaking.

(*3*) Open the 3M Petrifilm SALX Plate and streak onto the gel. Perform a single streak to obtain isolated colonies (Figure **2014.01**).

(*4*) Roll down the top film to close the 3M Petrifilm SALX Plate.

(*5*) Using a gloved hand (while practicing GLP to avoid cross-contamination and/or direct contact with the plate), gently apply a sweeping motion with even pressure onto the top film to remove any air bubbles in the inoculation area.

(*6*) Streak each enriched test portion onto a 3M Petrifilm SALX Plate and incubate at 41.5±1°C for 24±2 h in a horizontal position with the colored side up in stacks of no more than 20 plates.

G. Confirmation of 3M Petrifilm Salmonella Express Plates

(*1*) Using a permanent ultra-fine tip marker, circle at least five presumptive positive colonies (red to brown colonies with a yellow zone or associated gas bubble, or both) on the plate top film (*see* Table **2014.01D**).

(*2*) Lift the top film of the 3M Petrifilm SALX Plate and insert the 3M Petrifilm SALX Confirmation Disk by rolling it onto the gel to avoid entrapping air bubbles. Close the 3M Petrifilm SALX Plate. Using a gloved hand, gently apply a sweeping motion with even pressure onto the top film to remove any air bubbles in the inoculation area and ensure good contact between the gel and the 3M Petrifilm SALX Confirmation Disk.

(*3*) Incubate the 3M Petrifilm SALX System (plate and disk) at 41.5±1°C for 4–5 h in a horizontal position, right side up, in stacks of no more than 20 plates.

(*4*) Observe circled colonies for color change. Red/brown to green blue, blue, dark blue, or black confirms the colony as *Salmonella* spp. No color change indicates the colony is negative. If presumptive positive *Salmonella* colonies are not present, then report the results as *Salmonella* not detected in the matrix.

(*5*) Transfer typical colonies from 3M Petrifilm SALX Plate to TSI/LIA slants. Incubate 35±1°C for 24±2 h.

(*6*) Confirm a minimum of one typical colony per test portion with biochemical/serological procedures prescribed by the current versions of the USDA/FSIS-MLG or FDA/BAM reference methods.

Results of Collaborative Study

In this collaborative study, the 3M Petrifilm SALX System was compared to the USDA/FSIS-MLG 4.07 reference method for raw ground beef and to the FDA/BAM Chapter 5 reference method for dry dog food. A total of 17 laboratories throughout the United States participated in this study, with 15 laboratories submitting data for the raw ground beef and 14 laboratories submitting data for the dry dog food as presented in Table 1. Results of the heat stress analysis for the dry dog food inocula are presented in Table 2. Each laboratory analyzed 36 test portions for each method: 12 inoculated with a high level of *Salmonella*, 12 inoculated with a low level of *Salmonella*, and 12 uninoculated controls. A background screen of the matrix indicated an absence of indigenous *Salmonella* species. As per criteria outlined in Appendix J of the AOAC Validation Guidelines, fractional positive results were obtained for both matrices. For each matrix, the actual level of *Salmonella* was determined by MPN determination on the day of initiation of analysis by the coordinating laboratory. The individual laboratory and sample results are presented in Tables 3–4. Tables **2014.01A** and **B** summarize the collaborative study results for each matrix tested, including POD statistical analysis (7). Detailed results for each laboratory are presented in Appendix Tables 1–4 and Appendix Figures 1–8. The result for each collaborating laboratory's APC analysis for each matrix is presented in Appendix Table 5.

Raw Ground Beef (25 g Test Portions)

Raw ground beef test portions were inoculated at low and high levels and were analyzed (Table 3) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. Seventeen laboratories participated in the analysis of this matrix and the results of 14 laboratories were included in the statistical analysis. Laboratories 4, 6, and 9 reported that there were specific protocol deviations and therefore results from these laboratories were excluded from statistical analysis. The MPNs obtained for this matrix, with 95% confidence intervals, were 0.77 MPN/test portion (0.57, 0.88) for the low level and 4.67 MPN/test portion (3.38, 6.44) for the high

Table 1. Participation of each collaborating laboratory[a]

Lab	Raw ground beef (25 g test portions)	Dry dog food (375 g test portions)
1	Y	Y
2	Y	Y[b]
3	Y	Y
4	Y[b]	Y[b]
5	Y	Y
6	Y[b]	Y[b]
7	Y	Y
8	Y	Y
9	Y[b]	Y
10	Y	Y
11	Y	Y
12	Y	Y
13	Y	Y
14	Y	Y[b]
15	Y	Y
16	Y	Y
17	Y	N

[a] Y = Collaborator analyzed the food type and N = collaborator did not analyze the food type.

[b] Results were not used in statistical analysis due to deviation of testing protocol or laboratory error.

level. For the 3M Petrifilm SALX System, one test portion was confirmed positive by the traditional confirmation that was confirmed negative by the alternative confirmation. For all other test portions, no difference was observed between confirmation of samples using the alternative confirmation procedure and the traditional reference method confirmation procedure.

For the high level, 168 out of 168 test portions were reported as presumptive positive by the 3M Petrifilm SALX System with all test portions confirming positive by both the traditional and alternative confirmation methods. For the low level, 85 out of 168 test portions were reported as presumptive positive by the 3M Petrifilm SALX System, with 83 test portions confirming positive by both the traditional and alternative confirmation procedures. For the uninoculated controls, 2 out of 168 samples produced a presumptive positive result by the 3M Petrifilm SALX System method with one of the two presumptive positive samples confirming positive by the traditional reference method. All other test portions were negative. For test portions analyzed by the USDA/FSIS-MLG method, 167 out of 168 high inoculum and 86 out of 168 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 168 test portions confirmed positive.

Table 2. Heat-stress injury results

Matrix	Test organism[a]	CFU/XLD (selective agar)	CFU/TSA (Non-selective agar)	Degree injury
Dry dog food	*Salmonella* Poona NCTC 4840	3.0×10^8	9.0×10^8	77.7%

[a] NCTC = National Collection of Type Cultures.

For the low-level inoculum, a $dLPOD_C$ value of –0.02 (–0.13, 0.09) was obtained between the 3M Petrifilm SALX System using both confirmatory procedures and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.01 (–0.10, 0.12) was obtained between presumptive and confirmed 3M Petrifilm SALX System results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of 0.01 (–0.02, 0.03) was obtained between the 3M Petrifilm SALX System using both confirmatory procedures and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.00 (–0.02, 0.02) was obtained between presumptive and confirmed 3M Petrifilm SALX System results for both confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the uninoculated control level, $dLPOD_C$ values of 0.01 (–0.02, 0.03) and 0.00 (–0.02, 0.02) were obtained between the 3M Petrifilm SALX System using the traditional and alternative confirmation procedures, respectively, and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.01 (–0.02, 0.04) and 0.01 (–0.01, 0.04) was obtained between presumptive and confirmed 3M Petrifilm SALX System results using the traditional and alternative confirmation procedures, respectively. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Results of the POD statistical analysis are presented in Table **2014.01A** and Appendix Tables 1–2 and Appendix Figures 1–4.

Dry Dog Food (375 g Test Portions)

Dry dog food test portions were inoculated at low and high levels and were analyzed (Table 4) for the detection of *Salmonella* spp. Uninoculated controls were included in each analysis. Sixteen laboratories participated in the analysis of this matrix and the results of 12 of the laboratories were included in the statistical analysis. Two laboratories, 4 and 6, were unable to initiate sample testing at the start of the evaluation due to equipment malfunction or a delay in receiving their samples and therefore did not analyze any test portions. Two additional laboratories, 2 and 14, reported deviations from the testing protocol and therefore results from these laboratories were excluded from statistical analysis. The MPN obtained for this matrix, with 95% confidence intervals, were 0.69 MPN/test portion (0.54, 0.86) for the low level and 5.42 MPN/test portion (3.53, 8.30) for the high level. For the 3M Petrifilm SALX System, one test portion was confirmed positive by the traditional confirmation that was confirmed negative by the alternative confirmation. For all other test portions, no difference was observed between confirmation of samples using the alternative confirmation procedure and the traditional reference method confirmation procedure.

For the high level, 142 out of 144 test portions were reported as presumptive positive by the 3M Petrifilm SALX System with

Table 3. Individual collaborator results for raw ground beef (25 g test portions)[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
3M Petrifilm SALX System[b]																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	–	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	+	+	–	+	–[d]	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
4[e]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6[e]	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	–	–	–	-	+	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	-	+	–	–	–	–	–	–	–	–	–	–	–	–	–
9[e]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
10	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–[d]	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	+	–	+	+	+	–	+	–	–	–	–	+[c]	–	–	–	–	–	–	–	–
16	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–[d]	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
USDA/FSIS-MLG[d]																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
2	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
4[e]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
6[e]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9[e]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	
10	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–

Table 3. (*continued*)

Lab	High-level test portions												Low-level test portions												Uninoculated test portions											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
17	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] + = *Salmonella* spp. were detected in samples and – = *Salmonella* spp. were not detected in sample.

[b] Confirmed results from alternative and traditional confirmation were identical for each test portion except where noted.

[c] Sample confirmed positive by traditional method was negative by alternative confirmation.

[d] Sample was presumptive positive but confirmed negative.

[e] Results were not used in statistical analysis due to deviation of testing protocol laboratory error.

141 test portions confirming positive by both the traditional and alternative confirmation methods. For the low level, 82 out of 144 test portions were reported as presumptive positive by the 3M Petrifilm SALX System, with 82 test portions confirming positive by the traditional confirmation procedure and 81 test portions confirming positive by the alternative confirmation procedure. For the uninoculated controls, 0 out of 144 samples produced a presumptive positive result by the 3M Petrifilm SALX System method with 0 samples confirming positive by the traditional reference method. All other test portions were negative. For test portions analyzed by the FDA/BAM Chapter 5 Method, 144 out of 144 high inoculum and 71 out of 144 low inoculum test portions confirmed positive. For the uninoculated controls, 0 out of 144 test portions confirmed positive.

For the low-level inoculum, a $dLPOD_C$ value of 0.08 (–0.07, 0.22) and 0.07 (–0.07, 0.21) was obtained between the 3M Petrifilm SALX System with the traditional confirmation and alternative confirmation procedures, respectively, and the FDA/BAM method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods.

A $dLPOD_{CP}$ of 0.00 (–0.14, 0.14) and 0.01 (–0.18, 0.22) was obtained between presumptive and confirmed 3M Petrifilm SALX System results for the traditional and alternative confirmation procedures, respectively. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the high-level inoculum, a $dLPOD_C$ value of –0.02 (–0.06, 0.01) was obtained between the 3M Petrifilm SALX System with the traditional and alternative confirmation procedures and the FDA/BAM method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.01 (–0.03, 0.05) was obtained between presumptive and confirmed 3M Petrifilm SALX System results for the traditional and alternative confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results.

For the uninoculated control level, $dLPOD_C$ values of 0.00 (–0.03, 0.03) were obtained between the 3M Petrifilm SALX System using the traditional and alternative confirmation procedures and the FDA/BAM method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ of 0.03 (–0.03, 0.03) was obtained between presumptive and confirmed 3M Petrifilm SALX System results using the traditional and alternative confirmation procedures. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Results of the POD statistical analysis are presented in Table **2014.01A**, and Appendix Tables 3–4 and Appendix Figures 5–8.

Discussion

No negative feedback was reported to the Study Directors from the collaborating laboratories in regard to the performance of the 3M Petrifilm SALX System. For the analysis of the raw ground beef test portions by the 3M Petrifilm SALX System, three false-positive samples were obtained. For the analysis of the dry dog food, two false-positive samples and two false-negative samples were obtained.

Table 4. Individual collaborator results for dry dog food (375 g test portions)[a]

	High-level test portions												Low-level test portions												Uninoculated test portions											
Lab	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
3M Petrifilm SALX System[b]																																				
1	+	+	+	–	+	+	+	+	+	+	+	–[d]	+	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–[d]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	+	+	+	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4[f]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	+	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
6[f]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7	+	+	+	+	+	+	+	+[e]	+	+	+	+	–	+	–	–	+[e]	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	+	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
14[f]	+	+	+	+	+	+	+	+	+	+	–[d]	+	–[d]	+	–[d]	–[d]	–[d]	–	–[c]	+	–	+	+	–	–	+	–	–[d]	–	–	–	+	–	–[d]	–	–[d]
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–[d]	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
17	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
FDA/BAM																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
2[f]	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	+	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
4[f]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
6[f]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
8	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	+	–	+	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
9	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–
10	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	–	–	–	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
11	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	–	–	+	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 4. (*continued*)

Lab	High-level test portions												Low-level test portions												Uninoculated test portions											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
12	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	–	+	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–
14[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
15	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–	+	–	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–
16	–	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+	–	–	–	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–
17	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

[a] + = *Salmonella* spp. were detected in samples, – = *Salmonella* spp. were not detected in sample, and n/a = laboratory did not participate in this matrix or results were not received.

[b] Confirmed results from alternative and traditional confirmation were identical for each test portion unless noted.

[c] Sample was presumptive positive and confirmed negative by traditional confirmation but confirmed positive by alternative.

[d] Sample was presumptive positive but confirmed negative.

[e] Sample was presumptive negative but confirmed positive using the traditional confirmation.

[f] Results were not used in statistical analysis due to deviation of testing protocol laboratory error.

For the dry dog food, some laboratories reported high amounts of atypical growth on varying test portions and no background growth on other test portions. Because over 600 lbs of pet food was used in the evaluation, variability in the level of competing microflora among individual samples may have led to these discrepancies. Laboratory 7, which reported the two false-negative results for the dry dog food, indicated that a significant amount of atypical growth was observed on the 3M Petrifilm SALX Plate and believed it may have contributed to the difficulty in isolating *Salmonella* from those two samples.

The false-positive results observed for both matrices may have been the result of misidentification of typical colonies due to the misinterpretation of colony color (only five false positives out of 972 test portions) on the 3M Petrifilm SALX Plate. Additional experience with the method may eliminate some analyst uncertainty when selecting colonies believed to be presumptive positive for *Salmonella*. Because presumptive colonies are verified by placing the 3M Petrifilm SALX Confirmation Disk onto the 3M Petrifilm SALX Plate, no additional follow-up to verify if the correct colony was selected was possible by testing at the coordinating laboratory. For the raw ground beef, Laboratory 15 identified a presumptive positive colony in its uninoculated test portions, which was confirmed positive by the reference method. The data from this laboratory were included in the statistical analysis. Using the POD statistical model, no significant difference in the number of positive results obtained between the two methods being compared was observed at both the low and high inoculum levels for both matrices. Additionally, no significant difference was observed between presumptive and confirmed results for the candidate method.

Recommendations

It is recommended that the 3M Petrifilm *Salmonella* Express (SALX) System be adopted as Official First Action status for the detection of *Salmonella* in raw ground beef (25 g), raw ground chicken (25 g), pasteurized liquid whole egg (100 g), raw ground pork (25 g), cooked chicken nuggets (325 g), frozen uncooked shrimp (25 g), fresh bunched spinach (25 g), dry dog food (375 g), and stainless steel.

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AOAC Official Method 2014.05
Enumeration of Yeast and Mold in Food
3M™ Petrifilm™ Rapid Yeast and Mold Count Plate
First Action 2014

[Applicable to the enumeration of yeast and mold in the following high-water activity matrices: yogurt, frozen bread dough, fermented salami, sour cream, ready-made pie, raw frozen ground beef patties (77% lean), ready-to-eat deli sandwiches, sliced apples, and the following low-water activity matrices: raw almonds and dehydrated soup.]

See Tables **2014.05A** and **2014.05B** for a summary of results of the collaborative study. The result for each collaborating laboratory's aerobic plate count analysis for each matrix is shown in Table **2014.05C**.

See Tables 2-9 in the *J. AOAC Int*. paper for detailed results of the collaborative study.

A. Principle

The 3M™ Petrifilm™ Rapid Yeast and Mold Count (RYM) Plate is a sample-ready culture medium system, which contains nutrients supplemented with antibiotics, a cold-water-soluble gelling agent, and an indicator system that facilitates yeast and mold enumeration. 3M Petrifilm RYM Count Plates are used for the enumeration of yeast and mold in as little as 48 h in the food and beverage industries. 3M Food Safety is certified to ISO (International Organization for Standardization) 9001 for design and manufacturing.

B. Apparatus and Reagents

(**a**) *3M Petrifilm RYM Count Plate.*–25 plates/pouch; two pouches/box (3M Food Safety, St. Paul, MN, USA).

(**b**) *Sterile diluents.*--0.1% peptone water.

(**c**) *Pipets.*--Capable of 1000 µL or a serological pipet.

(**d**) *Sterile pipet tips.*--Capable of 1000 µL.

(**e**) *Stomacher.*--Seward or equivalent.

(**f**) *Filter stomacher bags.*--Seward or equivalent.

(**g**) *3M™ Petrifilm™ Flat Spreader.*

(**h**) *Incubators.*–Capable of maintaining 25 ± 1°C and 28 ± 1°C and having a solid front to maintain a dark interior.

(**i**) *Refrigerator.*--Capable of maintaining 2-8°C, for storing the 3M Petrifilm RYM Plates.

(**j**) *L-shaped spreaders.*

(**k**) *Standard colony counter or illuminated magnifier.*

C. General Instructions

(**a**) Store unopened 3M Petrifilm RYM Plate pouches refrigerated or frozen (-20 to 8°C/-4 to 46°F). Just prior to use, allow unopened pouches to come to room temperature before opening [20-25°C/<60% relative humidity (RH)]. Return unused 3M Petrifilm RYM Plates to pouch. Seal by folding the end of the pouch over and applying adhesive tape. To prevent exposure to moisture, do not refrigerate opened pouches. Store resealed pouches in a cool dry place (20-25°C/<60% RH) for no longer than 4 weeks. It is recommended that resealed pouches of 3M Petrifilm RYM Plates be stored in a freezer (*see* below) if the laboratory temperature exceeds 25°C (77°F) and/or the laboratory is located in a region where the RH exceeds 60% (with the exception of air-conditioned premises).

(**b**) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

D. Safety Precautions

After use, the diluents and 3M Petrifilm RYM Count Plates may contain microorganisms that may be a potential biohazard as several foodborne molds have the ability to produce toxic metabolites known as mycotoxins. If further identification of a mold species is required, appropriate personal protective equipment (PPE) should be used when top film is retracted and exposure to spores or mycotoxins may occur. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal. For information on potential biohazards, reference *Biosafety in Microbiological and Biomedical Laboratories*, 5th Ed., Section VIII-B: Fungal Agents.

The 3M Petrifilm RYM Count plates contain chloramphenical and chlortetracycline, potent broad spectrum antibiotic drugs commonly used in yeast and mold enumeration. The drug, when used in humans, is associated with many toxic effects. Care should be taken to avoid coming into direct contact with the gel on the plates.

E. Sample Preparation

(*1*) Aseptically prepare a 1:10 dilution of each test portion.

Dairy products.--Pipet 11 mL or weigh 11 g of sample into 99 mL sterile 0.1% peptone water. Shake 25 times to homogenize.

All other foods.–Weigh out 25 g of sample from test portion into a sterile stomacher bag and dilute with 225 mL of 0.1 % peptone water; stomach at high speed to homogenize.

(*2*) Prepare 10-fold serial dilutions in 0.1% peptone water.

(*3*) Place a 3M Petrifilm RYM Count Plate on a flat, level surface for each dilution to be tested.

(*4*) Lift the top of the film. Dispense 1 mL of each dilution onto the center of the bottom film of each plate.

(*5*) Roll the film down onto the sample.

(*6*) Place the 3M Petrifilm Flat Spreader (Cat. No. 6425) on the center of the plate. Press gently on the center of the spreader to distribute the sample evenly. Spread the inoculum over the entire 3M Petrifilm RYM Count Plate growth area before the gel is formed. *Do not slide the spreader across the film.*

(*7*) Remove the spreader and leave the plate undisturbed for at least 1 min to permit the gel to form.

(*8*) Incubate the 3M Petrifilm RYM Count Plates at 25 or 28°C in a horizontal position with the clear side up in stacks of no more than 40. Enumerate plates after 48 h of incubation. If colonies appear faint, allow up to an additional 12 h of incubation time for enhanced interpretation. 3M Petrifilm RYM Count Plates can be counted using a standard colony counter with the use of a back light or an illuminated magnifier to assist with the estimated enumeration.

(*9*) Yeast colonies appear raised and small with defined edges. Colonies may appear pink/tan to blue/green in color.

(*10*) Mold colonies appear flat with a dark center and diffused edges. Colonies may appear blue/green to variable upon prolonged incubation. *See* Table **2014.05D** for yeast and mold appearance.

(*11*) The circular growth area is approximately 30 cm^2. Plates containing greater than 150 colonies can be either estimated or recorded as TNTC (too numerous to count). Estimation can only be done by counting the number of colonies in one or more representative squares and determining the average number per square. The average number can be multiplied by 30 to determine the estimated count per plate. If a more accurate count is required, the sample will need to be retested at higher dilutions. When the sample contains substantial amounts of mold, depending on the type of mold, the upper countable limit may be at user discretion.

(*12*) Food samples may occasionally show interference on the 3M Petrifilm RYM Count Plates, for example:

(*a*) Uniform blue background color (often seen from the organisms used in cultured products). These should not be counted as TNTC.

(*b*) Intense pinpoint blue specks (often seen with spices or granulated products).

(*c*) Report final results as colony-forming units/gram (CFU/g).

(*13*) If required, colonies may be isolated for further identification by direct microscopy or biochemical analysis. Lift the top film and pick the colony from the gel.

Reference: *J. AOAC Int*. (future issue)

Posted: March 12, 2015

Table 2014.05A. Interlaboratory study results of 3M Petrifilm RYM vs FDA-BAM and ISO 21527 methods for frozen raw ground beef patties

3M Petrifilm RYM method							FDA-BAM/ISO 21527 methods[a]						*p*-Value[d]	Difference of means	Reverse transformed mean difference[e]
Matrix	**Lot**	***N*[b]**	**Mean[c]**	**s_r**		**s_R**	**Lot**	***N***	**Mean**	**s_r**		**s_R**			
Frozen raw ground beef patties (25°C, 48 h)	Control	11(0)	<1.00	-		-	Control	11(0)	<1.00	-		-	-	-	-
	Low	11(0)	2.12	0.41		0.41	Low	11(1)	2.07	0.36		0.38	0.5323	0.05	14.34
	Medium	11(0)	3.52	0.10		0.10	Medium	11(0)	3.47	0.09		0.11	0.1637	0.05	360.10
	High	11(0)	4.65	0.13		0.14	High	11(0)	4.59	0.10		0.14	0.2266	0.06	5763.84
Frozen raw ground beef patties (25°C, 60 h)	Control	11(0)	<1.00	-		-	Control	11(0)	<1.00	-		-	-	-	-
	Low	11(0)	2.14	0.36[f]		0.37	Low	11(1)	2.07	0.36		0.38	0.3773	0.07	20.55
	Medium	11(0)	3.52	0.10		0.10	Medium	11(0)	3.47	0.09		0.11	0.1573	0.05	360.10
	High	11(0)	4.65	0.14		0.15	High	11(0)	4.59	0.10		0.14	0.1750	0.06	5763.84
Frozen raw ground beef patties (28°C, 48 h)	Control	11(0)	<1.00	-		-	Control	11(0)	<1.00	-		-	-	-	-
	Low	11(0)	2.17	0.29[f]		0.30	Low	11(1)	2.07	0.36		0.38	0.1391	0.10	30.42
	Medium	11(0)	3.53	0.10		0.10	Medium	11(0)	3.47	0.09		0.11	0.0824	0.06	437.23
	High	11(0)	4.67	0.08[f]		0.11	High	11(0)	4.59	0.10		0.14	0.0966	0.08	7869.00
Frozen raw ground beef patties (28°C, 60 h)	Control	11(0)	<1.00	-		-	Control	11(0)	<1.00	-		-	-	-	-
	Low	11(0)	2.16	0.29[f]		0.29	Low	11(1)	2.07	0.36		0.38	0.1843	0.09	27.05
	Medium	11(0)	3.53	0.09		0.10	Medium	11(0)	3.47	0.09		0.11	0.1095	0.06	437.23
	High	11(0)	4.67	0.08[f]		0.11	High	11(0)	4.59	0.10		0.14	0.1088	0.08	7869.00

[a] Samples were analyzed by harmonized FDA-BAM Chapter 18 and ISO 21527 methods using 0.1% peptone as the sample diluent.

[b] *N* = Number of laboratories that reported complete results. Outliers are in parentheses.

[c] Log_{10} yeast and mold CFU/g.

[d] Significant difference ($p < 0.05$).

[e] Results presented as CFU/g.

[f] Results indicate that the candidate method is more repeatable than the reference methods. s_r = Repeatability standard deviation; s_R = reproducibility standard deviation.

Table 2014.05B. Interlaboratory study results of 3M Petrifilm RYM vs FDA-BAM and ISO 21527 methods for raw almonds																
3M Petrifilm RYM method								FDA-BAM/ISO 21527 methods[a]								
Matrix	Lot	*N*[b]	Mean[c]	s_r		s_R		Lot	*N*	Mean	s_r		s_R		*p*-Value[d]	Difference of means	Reverse transformed mean difference[e]
Raw almonds (25°C, 48 h)	Control	12(0)	<1.00	-		-		Control	12(0)	<1.00	-		-		-	-	-
	Low	14(0)	1.45	0.17[f]		0.26		Low	14(0)	1.55	0.19		0.34		0.4165	0.10	-7.30
	Medium	14(1)	2.12	0.26		0.39		Medium	14(0)	2.21	0.20		0.24		0.3322	0.09	-30.36
	High	14(2)	3.00	0.18		0.49		High	14(1)	3.08	0.12		0.31		0.2833	0.08	-202.26
Raw almonds (25°C, 60 h)	Control	12(0)	<1.00	-		-		Control	12(0)	<1.00	-		-		-	-	-
	Low	14(0)	1.53	0.23		0.28		Low	14(0)	1.55	0.19		0.34		0.8391	0.02	-1.60
	Medium	14(0)	2.20	0.21		0.27		Medium	14(0)	2.21	0.20		0.24		0.7789	0.01	-3.69
	High	14(2)	3.04	0.18		0.41		High	14(1)	3.08	0.12		0.31		0.5418	0.04	-105.79
Raw almonds (28°C, 48 h)	Control	12(0)	<1.00	-		-		Control	12(0)	<1.00	-		-		-	-	-
	Low	14(0)	1.58	0.16[f]		0.21		Low	14(0)	1.55	0.19		0.34		0.7381	0.03	2.54
	Medium	14(0)	2.17	0.17[f]		0.29		Medium	14(0)	2.21	0.20		0.24		0.6139	0.04	-11.73
	High	14(2)	3.01	0.17		0.45		High	14(1)	3.08	0.12		0.31		0.3904	0.07	-178.97
Raw almonds (28°C, 60 h)	Control	12(0)	<1.00	-		-		Control	12(0)	<1.00	-		-		-	-	-
	Low	14(0)	1.60	0.17[f]		0.20		Low	14(0)	1.55	0.19		0.34		0.5474	0.05	4.33
	Medium	14(0)	2.21	0.17[f]		0.23		Medium	14(0)	2.21	0.20		0.24		0.9483	0.00	0.00
	High	14(2)	3.03	0.18		0.42		High	14(1)	3.08	0.12		0.31		0.4687	0.05	-130.75

[a] Samples were analyzed by harmonized FDA-BAM Chapter 18 and ISO 21527 methods using 0.1% peptone as the sample diluent.

[b] *N* = Number of laboratories that reported complete results. Outliers are in parentheses.

[c] Log_{10} yeast and mold CFU/g.

[d] Significant difference ($p < 0.05$).

[e] Results presented as CFU/g.

[f] Results indicate that the candidate method is more repeatable than the reference methods. s_r = Repeatability standard deviation; s_R = reproducibility standard deviation.

Table 2014.05C. Results of aerobic plate count for collaborating laboratories

Lab	Frozen raw ground beef, CFU/g	Raw almonds, CFU/g
1	3.8×10^2	6.0×10^1
2	1.1×10^3	6.0×10^2
3	<10	3.0×10^1
4	Not reported	Not reported
5	2.8×10^3	2.8×10^1
6	8.0×10^1	2.2×10^1
7	9.1×10^2	1.6×10^2
8	Not reported	Not reported
9	9.0×10^2	2.0×10^2
10	1.3×10^3	4.0×10^2
11	>2500	1.0×10^1
12	Not reported	7.0×10^1
13	9.5×10^1	1.0×10^1
14	7.3×10^2	2.3×10^2
15	3.7×10^2	8.0×10^1

Table 2014.05D. Appearance of yeast and mold on 3M Petrifilm RYM Plates	
Yeast	**Mold**
Small colonies	Large colonies
Colonies have defined edges	Colonies have diffused edges
Pink/tan to blue/green in color	Blue/green to variable upon prolonged incubation
Colonies appear raised (3-dimensional)	Colonies appear flat
Colonies have a uniform color	Colonies have a dark center with diffused edges

Evaluation of the 3M™ Petrifilm™ Rapid Yeast and Mold Count Plate for the Enumeration of Yeast and Mold in Food: Collaborative Study

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The 3M™ Petrifilm™ Rapid Yeast and Mold (RYM) Count Plate is a simple, ready to use chromogenic culture method for the rapid detection and enumeration of yeast and mold in food products. The 3M Petrifilm RYM Count Plate was compared to the FDA BAM Chapter 18 *Yeasts, Molds and Mycotoxins* and the ISO 21527:2008 *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds* – Part 1: *Colony count technique in products with water activity greater than 0,95* and Part 2: *Colony count technique in products with water activity less than or equal to 0,95* reference methods for raw almonds and raw frozen ground beef patties (77% lean). The 3M Petrifilm RYM Count Plate was evaluated using a paired study design in a multi-laboratory collaborative study following the current AOAC Validation Guidelines. Three (3) target contamination levels (low, 10-100 colony forming unit (CFU)/g; medium, 100-1,000 CFU/g; high 1,000-10,000 CFU/g) as well as an uninoculated control level (0 CFU/g) were evaluated for each matrix. Samples evaluated by the 3M Petrifilm RYM Count Plate were prepared in duplicate and incubated at both 25°C and 28°C. Plates at both temperatures were enumerated after 48 and 60 hours of incubation. No significant difference was observed between the 3M Petrifilm RYM Plate Count and the FDA BAM or ISO 21527 reference methods for each contamination level. No statistical differences were observed between samples analyzed by the 3M Petrifilm RYM Count Plate (at either 25°C or 28°C) and the reference methods. No statistical significant differences were observed between enumeration of colonies at 48 and 60 hours on the 3M Petrifilm RYM Count Plate and the reference methods.

Foodborne yeasts and mold constitute a large and diverse group of microorganisms. Due to their heterotrophic nature, these microorganisms are able to adapt and survive in a wide range of environmental conditions including acidic and alkaline conditions, temperatures ranging from 5 °C to 35°C and in low moisture products (<0.85$_{Aw}$) [1, 2]. Many of these species cause varying degrees of food decomposition leading to substantial economic loses [2]. Several foodborne molds, and possibly some yeasts, may also be hazardous to human health due to their ability to produce toxic metabolites [1]. The presence of these microorganisms in food commodities can indicate contamination of the product, inadequately cleaned food processing equipment or inadequate food storage facilities [2]. Cultural enumeration of yeasts and molds can take 5 to

7 days and the need for rapid enumeration is critical to the food industry. The 3M™ Petrifilm™ Rapid Yeast and Mold Count (RYM) Plate uses a cold-water-soluble gelling agent and an indicator system to facilitate the enumeration of yeast and molds after 48 hours of incubation.

The 3M Petrifilm RYM Count Plate allows for the simple and rapid enumeration of yeasts and molds in the food and beverage industries. Samples are diluted in 0.1% peptone water and a sample aliquot is plated onto the 3M Petrifilm RYM Count Plate. The sample aliquot is dispersed throughout the growth area and the plates can be incubated at 25°C - 28°C. Enumeration of colonies occurs in as little as 48 hours of incubation. If colonies appear faint, the plates can be incubated up to an additional 12 hours for enhanced interpretation. The 3M Petrifilm RYM Count Plate provides gridlines to assist with the enumeration.

Prior to the collaborative study, the 3M Petrifilm RYM Count Plate was validated according to AOAC Validation Guidelines [3] in a harmonized AOAC Performance Tested Method℠ (PTM) study. The objective of the PTM study was to demonstrate that the 3M Petrifilm RYM Count Plate accurately detected and enumerated yeasts and molds in selected foods as claimed by the manufacturer and that no difference in repeatability was observed between the 3M Petrifilm RYM Count Plate method and the reference methods. For the 3M Petrifilm RYM Count Plate PTM evaluation, ten (10) matrices were evaluated: yogurt (1.5% fat), sour cream, raw almonds, sliced apples, frozen bread dough, ready-made cherry pie, ready-to-eat deli sandwiches, dehydrated chicken noodle soup, fermented salami and raw frozen ground beef patties (77% lean).

All other PTM parameters (inclusivity, exclusivity, ruggedness, stability and lot-to-lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method was awarded PTM certification number 121301 on December 18, 2013.

The purpose of this collaborative study was to compare the 3M Petrifilm RYM Count Plate to a harmonized version of the U.S. Food and Drug Administration (FDA) Bacterial Analytical Manual (BAM) Chapter 18: Yeasts, Molds and Mycotoxins [1] and the ISO 21527:2008 *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds* – Part 1: *Colony count technique in products with water activity greater than 0,95* [4] and Part 2: *Colony count technique in products with water activity less than or equal to 0,95* [5] methods using 0.1% Peptone water as the diluent for raw almonds and raw frozen ground beef patties (77% lean).

Collaborative Study

Study Design

In this collaborative study, two matrices, raw frozen ground beef patties (77% lean) and raw almonds, were evaluated. The matrices were obtained from local retailers and screened for the presence of yeast and molds by the BAM and ISO reference methods. The raw frozen ground beef patties were artificially contaminated with a yeast, *Trichosporon mucoides* American Type Culture Collection (ATCC) 201382, and the almonds with a mold, *Aspergillus aculeatus* ATCC 56925. Four separate levels of contamination, including an uninoculated control level and 3 levels of artificial contamination, were evaluated for each matrix. The target for the three levels of artificial contamination was as follows: low, 10-100 colony forming unit (CFU)/g; medium, 100-1,000 CFU/g; high 1,000-10,000 CFU/g. Two replicate samples from each of the four levels were analyzed by both the candidate and reference methods. One set of paired samples (8 total) per matrix was sent to each laboratory for analysis by the 3M Petrifilm RYM Count Plate and the BAM and ISO reference methods. For both matrices, collaborators were sent an additional 60 g test portion and instructed to conduct a total aerobic plate count (APC) using 3M Petrifilm

Aerobic Count Plate (AOAC Official Method 990.12) [6] on the day samples were received as a quality measure to verify appropriate shipping conditions.

A detailed collaborative study packet outlining all necessary information related to the study including media preparation, test portion preparation and documentation of results was sent to each collaborating laboratory prior to the initiation of the study. A conference call prior to the initiation of the study was conducted to discuss the collaborative study packet and answer any questions from the participating laboratories.

Preparation of Inocula and Test Portions

The *Trichosporon mucoides* used in this evaluation was propagated in 10 mL of Brain Heart Infusion (BHI) broth from a frozen stock culture stored at -70°C at Q Laboratories, Inc. The broth was incubated for 48 ± 2 hours at 30 ± 1°C. Appropriate dilutions of the cultures were prepared in Butterfields Phosphate Buffer (PBW) based on previously established growth curves to obtain low, medium and high contamination levels. For the raw frozen ground beef patties, a bulk lot of the matrix was thawed, inoculated with the diluted liquid inoculum and mixed thoroughly by hand kneading to ensure an even distribution of microorganisms. The inoculated raw frozen ground beef patties samples were separated into 30 g test portions and held at -20 ± 2°C for 2 weeks when testing was initiated.

The *Aspergillus aculeatus* used in this evaluation was propagated on Potato Dextrose Agar (PDA) in a culture tissue flask from a frozen stock spore suspension stored at -70°C at Q Laboratories, Inc. The culture tissue flask was incubated for 7 days at 25 ± 1°C. The mold spore suspension was prepared by rinsing the culture tissue flask twice with 20 mL of 0.9% saline containing 0.05% Tween 20. Each suspension was centrifuged at 4,000 x *g* for 10 ± 0.5 minutes to pellet the spores and the supernatant was decanted. The two separate spore pellets were resuspended with 2.5 mL of PBW and combined. The spore suspension was combined with a cryoprotectant, reconstituted Non-Fat Dry Milk (NFDM), homogenized by vortex to mix and placed onto a freeze dry system for 72 ± 4 hours to lyophilize the culture. After the lyophilization process, appropriate dilutions of the culture were prepared in sterile NFDM powder to produce the low, medium and high contamination levels. A bulk lot of whole raw almonds were inoculated with the lyophilized inoculum and mixed thoroughly by hand mixing to ensure an even distribution of microorganisms. The inoculated almonds were separated into 30 g test portions and held at ambient temperature (24 ± 2°C) so that the organism had equilibrated for 2 weeks when testing was initiated.

The shipment conditions and hold times of the inoculated test materials were verified as a quality control measure prior to study initiation

Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3 digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by International Air Transport Association (IATA). Frozen raw ground beef patties samples were packed with cold packs to ensure the samples remained frozen (-20 ± 2°C) during shipment. Upon receipt, raw frozen ground beef patties samples were held at -20 ± 2°C until the following Monday when analysis was initiated. Raw almond samples were packed and shipped at ambient temperature. Upon receipt, samples were held at room temperature (24 ± 2 ^{o}C) until the following Monday when analysis was initiated. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as “temperature control”.

Participants were instructed to record the temperature of this portion upon receipt of the shipment, document results on the Sample Receipt Confirmation form provided and fax to the study director.

Test Portion Analysis

Collaborators followed the appropriate preparation and analysis protocol according to the method specified for each matrix. For both matrices, each collaborator received 8 test portions (2 high, 2 medium, 2 low and 2 uninoculated replicates). For the analysis of the each matrix by the 3M Petrifilm RYM Count Plate, a 25 g test portion was diluted with 225 mL of 0.1% Peptone Water and homogenized for 2 minutes. Ten-fold serial dilutions of each sample were prepared and a 1.0 mL aliquot of each dilution was plated onto 4 separate 3M Petrifilm RYM Count Plates. For each dilution, 2 of the plates were incubated at 25 ± 1°C and 2 of the plates were incubated at 28 ± 1°C. Plates were removed from incubation after 48 ± 2 hrs and typical yeast and mold colonies in the countable range (15-150) were enumerated using a standard colony counter. Plates containing greater than 150 colonies were either estimated or recorded as to numerous to count (TNTC). All plates were re-incubated at the appropriate temperatures for an additional 10-14 hours (to reach a total incubation time of 60 hours) and colonies were enumerated a second time in the same manner as the 48 hour time point.

Both matrices analyzed by the 3M Petrifilm RYM Count Plate were also analyzed using the BAM and ISO reference method in a paired study design. Serial dilutions for each sample were spread plated in triplicate onto Dichloran-rose Bengal Chloramphenicol (DRBC) agar (raw frozen ground beef patties) or Dichloran 18% Glycerol (DG18) agar (raw almonds). Agar plates were incubated for 5 days at 25 ±1°C and typical colonies in the countable range (10-150) were enumerated using a standard colony counter. Plates containing no colonies were re-incubated for 2 days and observed for typical growth.

Statistical Analysis

Each collaborating laboratory recorded the CFU/g results for the reference methods and the 3M Petrifilm RYM Count Plate on the electronic spreadsheet provided in the collaborator study outline. The data sheets were submitted to the study director at the end of each week of testing for analysis. The data from each duplicate plates (3M Petrifilm RYM Count Plate) or triplicate plates (BAM and ISO) were averaged. The averaged counts were converted into logarithms for data analysis. Outliers were identified using the Cochran and Grubbs' tests. A paired t-test was conducted to determine if the mean of replicate samples at each contamination level for each matrix was different between the 3M Petrifilm RYM Count Plate and the reference methods. [3]. A p-value greater than the standard alpha value of 0.05 indicated no statistical difference between the two methods. The repeatability (s_r), reproducibility (s_R), relative standard deviation of repeatability (RSD_r) and relative standard deviation of reproducibility (RSD_R) of the 3M Petrifilm RYM Count Plate and reference methods were determined by the mean of the logarithm transformations of the counts for each contamination level of each matrix [7]. A lower standard deviation value indicated a greater propensity for repeatability of a method. In addition, the difference of means and reverse transformed difference of mean for each contamination level was determined. [3].

AOAC Official Method 2014.xxx
Enumeration of Yeast and Mold in Food
3M™ Petrifilm™ Rapid Yeast and Mold Count Plate
First Action 2014

(Applicable to the enumeration of yeast and mold in the following high water activity matrices: yogurt, frozen bread dough, fermented salami, sour cream, ready-made pie, raw frozen ground beef patties (77% lean), ready-to-eat deli sandwiches, sliced apples, and the following low water activity matrices: raw almonds and dehydrated soup)

*See Tables **2014.1** and **2014.2** for a summary of results of the collaborative study.*
See Tables 2-9 for detailed of results of the collaborative study

A. Principle

The 3M Petrifilm Rapid Yeast and Mold Count (RYM) Plate is a sample-ready culture medium system which contains nutrients supplemented with antibiotics, a cold-water-soluble gelling agent, and an indicator system that facilitates yeast and mold enumeration. 3M Petrifilm RYM Count Plates are used for the enumeration of yeast and mold in as little as 48 hours in the food and beverage industries 3M™ Food Safety is certified to ISO (International Organization for Standardization) 9001 for design and manufacturing.

B. Apparatus and Reagents

B. (a) *3M™ Petrifilm™ Rapid Yeast and Mold Count Plate* – 25 plates/pouch 2 pouches /box. Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).

B. (b) *Sterile Diluent*- 0.1% peptone water

B. (c) *Pipettes*- capable of pipetting 1,000 µL or a serological pipette

B. (d) *Sterile pipette tips*- capable of 1,000 µL

B. (e) *Stomacher*- Seward or equivalent

B. (f) *Filter Stomacher bags*- Seward or equivalent

B. (g) *3M™ Petrifilm™ Flat Spreader*

B. (h) *Incubators* – Capable of maintaining 25 ± 1°C and 28 ± 1°C and having a solid front to maintain a dark interior

B. (i) *Refrigerator* - capable of maintaining 2-8° C, for storing the 3M Petrifilm RYM plates

B. (j) L shaped spreaders

B. (k) Standard Colony Counter or Illuminated Magnifier

C. General Instructions

C. (a) Store the 3M Petrifilm RYM Plates at 2-8°C. After opening the 3M Petrifilm RYM Plate pouches, seal the pouch and store at ambient temperature, less than 60% relative humidity. Post incubation 3M Petrifilm RYM Plates can be stored at -10°C to -20°C for up to 3 days. Place the 3M Petrifilm Flat Spreader on the center of the plate. Prior to use, place the plates on a flat surface for 1 hour at room temperature (20-25°C/< 60% RH) and protected from light to allow the gel to form. Hydrated plates can be stored at room temperature for up to 8 hours before use.

C. (b) Follow all instructions carefully. Failure to do so may lead to inaccurate results

C. (c) Safety Precautions

After use, the diluents and 3M Petrifilm RYM Count Plates may contain microorganisms that may be a potential biohazard as several foodborne molds have the ability to produce toxic metabolites known as mycotoxins. If further identification of a mold species is required, appropriate PPE's should be utilized when top film is retracted and exposure to spores or mycotoxins may occur. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal. For information on potential biohazards, reference Biosafety in Microbiological and Biomedical Laboratories, 5th edition, Section VIII-B: Fungal Agents.

The 3M Petrifilm RYM Count plates contain chloramphenicol, a potent, broad spectrum antibiotic drug. The drug, when used in humans, is associated with many toxic effects. Care should be taken to avoid coming into direct contact with the gel on the plates.

D. Sample Preparation

1. Aseptically prepare a 1:10 dilution of each test portion

Dairy products- Pipet 11 mL or weigh 11 g of sample into 99 mL of sterile 0.1% peptone water shake 25 times to homogenize

All other foods – Weigh out 25 g of sample from test portion into a sterile stomacher bag and dilute with 225 mL of 0.1 % peptone water; stomach at high speed to homogenize

2. Prepare 10 fold serial dilutions in 0.1% peptone water

3. Place a 3M Petrifilm RYM Count Plate on a flat, level surface for each dilution to be tested

4. Lift the top of the film with the pipette perpendicular dispense 1 mL of each dilution onto the center of the bottom film of each plate

5. Roll the film down onto the sample

6. Place the 3M Petrifilm Flat Spreader (6425) on the center of the plate. Press gently on the center of the spreader to distribute the sample evenly. Spread the inoculum over the entire 3M Petrifilm RYM Count Plate growth area before the gel is formed. **Do not slide the spreader across the film**

7. Remove the spreader and leave the plate undisturbed for at least one minute to permit the gel to form

8. Incubate the 3M Petrifilm RYM Count Plates at either 25°C - 28°C in a horizontal position with the clear side up in stacks of no more than 40. Enumerate plates after **48** of incubation**.** If colonies appear faint, allow up to an additional 12 hours of incubation time for enhanced interpretation. 3M Petrifilm RYM Count Plates can be counted using a standard colony counter with the use of a back light or an illuminated magnifier to assist with the estimated enumeration.

9. Yeast colonies appear raised, small with defined edges. Colonies may appear Pink –tan to blue green in color

10. Mold colonies appear flat with a dark center and diffused edges. Colonies may appear Blue/green to variable upon prolonged incubation.

YEAST	**MOLD**
Small colonies	Large colonies

Colonies have defined edges	Colonies have diffuse edges
Pink-tan to blue-green in color	Blue/green to variable upon prolonged incubation
Colonies appear raised (3 dimensional)	Colonies appear flat
Colonies have a uniform color	Colonies have a dark center with diffused edge

11. The circular growth area is approximately 30 cm^2. Plates containing greater than 150 colonies can be either estimated or recorded as TNTC. Estimation can only be done by counting the number of colonies in one or more representative squares and determining the average number per square. The average number can be multiplies by 30 to determine the estimated count per plate. If a more accurate count is required the sample, will need to be retested at higher dilutions. When the sample contains substantial amounts of mold, depending on the type of mold, the upper countable limit may be at user discretion.

12. Food samples may occasionally show interference on the 3M Petrifilm RYM Count Plates for example:

a. Uniform blue background color (Often seen from the organisms used in cultured products) these should not be counted as TNTC.

b. Intense pinpoint blue specs (often seen with spices or granulated products).

c. Report final results as colony forming units/gram (cfu/g)

13. If required, colonies may be isolated for further identification by direct microscopy or biochemical analysis. Lift the top film and pick the colony from the gel.

Results of the Collaborative Study

In this collaborative study, the 3M Petrifilm RYM Count Plate was compared to two reference methods for enumerating total yeast and mold: FDA BAM Chapter 18 and ISO 21527 Part 1 or Part 2. A total of 15 laboratories throughout the United States participated, with 11 laboratories submitting valid data for the frozen raw ground beef patties and 14 laboratories submitting valid data for the raw almonds as presented in Table 1. For the frozen raw ground beef patties, laboratories 4 (failed to enumerate 3M RYM Petrifilm plates at 60 hours), 6 and 8 (plated the reference method samples in duplicate and not the required triplicate plating) and 12 (plated the 3M Petrifilm RYM plates and reference method plates on two different days (enrichments were held at 2-8^oC overnight and the Petrifilm RYM plates were plated 24 hours after the reference method plates)) reported deviations from the protocol; ; and were therefore excluded from statistical analysis. For the raw almonds, laboratory 4 reported a deviation from the protocol (failure to enumerate 3M RYM Petrifilm Plates at 60 hours) and was therefore excluded from the statistical analysis.

The 3M Petrifilm RYM Count Plate results along with FDA BAM and ISO results reported by each lab were converted to logarithmic values for statistical analysis and were plotted using a Youden's plot (see Figures 1-4). The $\log_{10}$ individual lab results are presented in Tables 2-9. Using the Youden's plots, an initial review of the data to determine outliers was conducted by observing the mean replicate results for each laboratory at each contamination level for each matrix. The transformed data was than statistically analyzed for outliers by the Cochran and Grubb's tests. No evidence of physical cause or suspicion of cause was noted, so all outliers identified were included in the statistical analysis. A paired t-test, the difference of means, and

the reverse transformed mean difference \were calculated on each contamination level for each matrix to determine if a statistical significant difference existed between the methods. Repeatability (s_r), reproducibility (s_R), standard deviation of repeatability (RSD_r) and standard deviation of reproducibility (RSD_R) were determined for each contamination level for both the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 methods. The results of the interlaboratory data analyses are presented in Tables 2014.1 and 2014.2. The result for each collaborating laboratory's aerobic plate count analysis for each matrix is presented in Table 2014.3.

Frozen Raw Ground Beef Patties (77% lean)

Frozen raw ground beef patties test portions were inoculated at a low, medium and high contamination level and were analyzed (Tables 2-5) for the enumeration of yeast and mold. Uninoculated controls were included in each analysis. Fifteen laboratories participated in the analysis of this matrix.

3M RYM Count Plate incubated at 25°C and enumerated at 48 hours

The mean $\log_{10}$ counts of the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 results were compared statistically. One (1) laboratory in the FDA BAM/ISO 21527 low contamination level was identified as an outlier as defined by the Cochran's tests for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.

There were no statistically significant differences at the 5% level in log transformed means between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21257 methods, at any of the three contamination levels.

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3M RYM Count Plate incubated at 25°C and enumerated at 60 hours

The mean $\log_{10}$ counts of the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 results were compared statistically. One (1) laboratory in the FDA BAM/ISO 21527 low contamination level was identified as an outlier as defined by the Cochran's tests for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.

There were no statistically significant differences at the 5% level in log transformed means between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21257 methods, at any of the three contamination levels.

3M RYM Count Plate incubated at 28°C and enumerated at 48 hours

The mean $\log_{10}$ counts of the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 results were compared statistically. One (1) laboratory in the FDA BAM/ISO 21527 low contamination level was identified as an outlier as defined by the Cochran's tests for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.

There were no statistically significant differences at the 5% level in log transformed means between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21257 methods, at any of the three contamination levels.

3M RYM Count Plate incubated at 28°C and enumerated at 60 hours

The mean $\log_{10}$ counts of the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 results were compared statistically. One (1) laboratory in the FDA BAM/ISO 21527 low contamination level was identified as an outlier as defined by the Cochran's tests for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.

There were no statistically significant differences determined between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 methods at the 95% level or between the differences of means at all three contamination levels.

Raw Almonds

Raw almond test portions were inoculated at a low, medium and high contamination level and were analyzed (Tables 6-9) for the enumeration of yeast and mold. Uninoculated controls were included in each analysis. Fifteen laboratories participated in the analysis of this matrix.

3M RYM Count Plate incubated at 25°C and enumerated at 48 hours

The mean $\log_{10}$ counts of the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 results were compared statistically. One (1) laboratory was identified in the 3M Petrifilm RYM Count Plate medium contamination results as an outlier by the Cochran's test for outliers, one laboratory was identified in the FDA BAM/ISO 21527 high contamination level as an outlier by the Single Grubbs' test and two laboratories in the 3M Petrifilm RYM Count Plate high contamination level were identified as an outlier by the double Grubbs' test for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.

There were no statistically significant differences at the 5% level in log transformed means between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21257 methods, at any of the three contamination levels.

3M RYM Count Plate incubated at 25°C and enumerated at 60 hours

The mean $\log_{10}$ counts of the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 results were compared statistically. One (1) laboratory was identified in the FDA BAM/ISO 21527 high contamination level as an outlier by the Single Grubbs' test and two laboratories in the 3M Petrifilm RYM Count Plate high contamination level were identified as an outlier by the double Grubbs' test for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.

There were no statistically significant differences determined between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 methods at the 95% level or between the differences of means at all three contamination levels.

3M RYM Count Plate incubated at 28°C and enumerated at 48 hours

The mean $\log_{10}$ counts of the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 results were compared statistically. One (1) laboratory was identified in the FDA BAM/ISO 21527 high contamination level as an outlier by the Single Grubbs' test and two laboratories in the 3M Petrifilm RYM Count Plate high contamination level were identified as an outlier by the double Grubbs' test for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.

There were no statistically significant differences at the 5% level in log transformed means between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21257 methods, at any of the three contamination levels.

3M RYM Count Plate incubated at 28°C and enumerated at 60 hours

The mean $\log_{10}$ counts of the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21527 results were compared statistically. One (1) laboratory was identified in the FDA BAM/ISO 21527 high contamination level as an outlier by the Single Grubbs' test and two laboratories in the 3M Petrifilm RYM Count Plate high contamination level were identified as an outlier by the double Grubbs' test for outliers. However, no evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.
There were no statistically significant differences at the 5% level in log transformed means between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21257 methods, at any of the three contamination levels..

Discussion

No negative feedback was reported to the study directors from the collaborating laboratories in regards to the performance of the 3M Petrifilm RYM Count Plate. A few laboratories indicated difficulty in spreading the test portion liquid aliquot over the entire surface of the plate before the sample began to solidify in the Petrifilm medium. Several laboratories also indicated that the colonies were very easy to interpret due to their size and color. One laboratory indicated that the "Space saving benefits of Petrifilm over the traditional FDA-BAM method are readily apparent."

During the analysis of samples, several laboratories indicated deviating from the approved protocol and therefore, their data was subsequently removed from statistical analysis. No laboratories were removed from statistical analysis as a result of their data. For the raw ground beef four laboratories were removed from statistical analysis. Laboratory 12 was unable to enumerate samples by both the Petrifilm RYM Plate and reference methods on the same day. All samples were plated onto DRBC, then stored at 2-8°C overnight and plated onto the Petrifilm RYM plates the following day. Laboratories 6 and 8 prepared the reference method samples using duplicate plating and not the required triplicate plating. Laboratory 4 (for both raw ground beef and raw almonds) failed to enumerate the 3M RYM Petrifilm plates at the 60 hour time point. Due to these deviations, these laboratories were removed from statistical analysis.

There were no statistically significant differences at the 5% level in log transformed means between the 3M Petrifilm RYM Count Plate and FDA BAM/ISO 21257 methods for either matrix evaluated in this study, at any of the three contamination levels. . No statistically significant differences were observed between the 3M Petrifilm RYM Count Plate results when enumerated at 48 hours vs. 60 hours and compared to the FDA BAM/ISO 21527 methods. No statistically significant differences were also observed between 3M Petrifilm RYM Count Plates that were incubated at 25°C and 28°C when compared to the FDA BAM/ISO 21527 methods.

Based on the data presented, the repeatability values obtained at all incubation conditions and for all three contamination levels was generally similar between the two methods.

For raw frozen ground beef patties and raw almonds, the 3M Petrifilm RYM Count Plate was more repeatable than the FDA BAM/ISO 21527 reference methods for 1 of the 6 contamination levels analyzed at 25°C and 4 of the 6 contamination levels analyzed at 28°C.

Recommendations

It is recommended that the 3M Petrifilm Rapid Yeast and Mold Count (RYM) Plate be adopted as Official First Action status for the enumeration of yeast and molds in the following high water activity matrices: yogurt (1.5% fat), frozen bread dough, fermented salami, sour cream, ready-made pie, raw frozen ground beef patties (77%), ready-to-eat deli sandwiches, sliced apples and the following low water activity matrices: raw almonds and dehydrated soup.

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References

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(4) International Organization for Standardization 21527-1:2008 *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of yeasts and moulds – Part 1: Colony count technique in products with water activity greater than 0,95.* 2012

(5) International Organization for Standardization 21527-2:2008 *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of yeasts and moulds – Part 2: Colony count technique in products with water activity less than or equal to 0,95.* 2012

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Table 1: Participation of each Collaborating Laboratory[a]

Lab	Raw Ground Beef	Almonds
1	Y	Y
2	Y	Y
3	Y	Y
4	Y[b]	Y[b]
5	Y	Y
6	Y[b]	Y
7	Y	Y
8	Y[b]	Y
9	Y	Y
10	Y	Y
11	Y	Y
12	Y[b]	Y
13	Y	Y
14	Y	Y
15	Y	Y

[a] Y= Collaborator analyzed the food type; N= collaborator did not analyze the food type

[b] Results were not used in statistical analysis due to deviation of testing protocol or laboratory error

Table 2. Total Yeast and Mold Log_{10} counts for frozen raw ground beef patties by 3M Petrifilm RYM Count Plate after 48 hours incubation at 25°C vs. FDA BAM and ISO 21527 reference methods[a]

	Uninoculated Control				Low				Medium				High			
	Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]	
Lab	A[b]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	<1.00	<1.00	<1.00	<1.00	2.18	1.78	2.23	1.60	3.45	3.38	3.49	3.32	4.64	4.62	4.32	4.54
2	<1.00	<1.00	<1.00	<1.00	2.43	2.48	2.43	2.83	3.62	3.71	3.49	3.45	4.62	4.73	4.53	4.52
3	<1.00	<1.00	<1.00	<1.00	2.34	2.30	2.41	2.32	3.52	3.30	3.63	3.53	4.64	4.40	4.82	4.81
4[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	<1.00	<1.00	<1.00	<1.00	1.95	1.78	1.70	2.00	3.41	3.53	3.30	3.41	4.43	4.51	4.46	4.64
6[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	<1.00	<1.00	<1.00	<1.00	2.23	2.36	1.80	1.82	3.51	3.56	3.36	3.61	4.79	4.32	4.43	4.49
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	<1.00	<1.00	<1.00	<1.00	2.45	2.23	2.28	2.23	3.56	3.60	3.51	3.45	4.72	4.73	4.57	4.79
10	<1.00	<1.00	<1.00	<1.00	2.20	2.20	2.11	2.00	3.60	3.45	3.45	3.43	4.76	4.61	4.66	4.40
11	<1.00	<1.00	<1.00	<1.00	2.36	1.00	2.40	1.90	3.57	3.66	3.49	3.54	4.68	4.56	4.49	4.59
12[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	<1.00	<1.00	<1.00	<1.00	2.45	2.08	2.00	1.67	3.49	3.51	3.52	3.48	4.75	4.89	4.67	4.77
14	<1.00	<1.00	<1.00	<1.00	2.20	1.00	2.34[c]	1.00[c]	3.41	3.67	3.57	3.70	4.70	4.60	4.65	4.62
15	<1.00	<1.00	<1.00	<1.00	2.30	2.28	2.15	2.23	3.38	3.53	3.38	3.23	4.72	4.85	4.61	4.66

RYM= 3M Petrifilm Rapid Yeast and Mold method.

FDA BAM= FDA BAM Chapter 18 method for enumeration of yeasts, molds and mycotoxins.

ISO 21527= ISO 21527:2008 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds – Part 1: Colony count technique in products with water activity greater than 0,95 [4] and Part 2: Colony count technique in products with water activity less than or equal to 0,95

[a] Log_{10} yeast and mold CFU/g;

[b] A and B= Indicated duplicate test portions;

[c] Cochran's outlier

[d] Laboratory data not included in statistical analysis for all contamination levels

[e] FDA BAM and ISO 21257 reference method replicates were enumerated at 5 and 7 days (uninoculated test portions) of incubation at 25°C

Table 3. Total Yeast and Mold Log_{10} counts for frozen raw ground beef patties by 3M Petrifilm RYM Count Plate after 60 hours incubation at 25°C vs. FDA BAM and ISO 21527 reference methods[a]

	Uninoculated Control				Low				Medium				High			
	Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]	
Lab	A[b]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	<1.00	<1.00	<1.00	<1.00	2.18	1.78	2.23	1.60	3.45	3.40	3.49	3.32	4.62	4.61	4.32	4.54
2	<1.00	<1.00	<1.00	<1.00	2.43	2.48	2.43	2.83	3.62	3.71	3.49	3.45	4.62	4.73	4.53	4.52
3	<1.00	<1.00	<1.00	<1.00	2.30	2.26	2.41	2.32	3.56	3.30	3.63	3.53	4.68	4.40	4.82	4.81
4[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	<1.00	<1.00	<1.00	<1.00	1.95	1.78	1.70	2.00	3.41	3.53	3.30	3.41	4.43	4.51	4.46	4.64
6[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	<1.00	<1.00	<1.00	<1.00	2.23	2.36	1.80	1.82	3.51	3.56	3.36	3.61	4.79	4.32	4.43	4.49
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	<1.00	<1.00	<1.00	<1.00	2.45	2.23	2.28	2.23	3.56	3.58	3.51	3.45	4.72	4.75	4.57	4.79
10	<1.00	<1.00	<1.00	<1.00	2.20	2.20	2.11	2.00	3.60	3.45	3.45	3.43	4.76	4.61	4.66	4.40
11	<1.00	<1.00	<1.00	<1.00	2.36	1.30	2.40	1.90	3.56	3.57	3.49	3.54	4.68	4.56	4.49	4.59
12[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	<1.00	<1.00	<1.00	<1.00	2.45	2.32	2.00	1.67	3.51	3.51	3.52	3.48	4.76	4.92	4.67	4.77
14	<1.00	<1.00	<1.00	<1.00	2.20	1.00	2.34[c]	1.00[c]	3.41	3.68	3.57	3.70	4.70	4.62	4.65	4.62
15	<1.00	<1.00	<1.00	<1.00	2.30	2.28	2.15	2.23	3.38	3.53	3.38	3.23	4.72	4.85	4.61	4.66

RYM= 3M Petrifilm Rapid Yeast and Mold method.

FDA BAM= FDA BAM Chapter 18 method for enumeration of yeasts, molds and mycotoxins.

ISO 21527= ISO 21527:2008 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds – Part 1: Colony count technique in products with water activity greater than 0,95

[a] Log_{10} yeast and mold CFU/g;

[b] A and B= Indicated duplicate test portions;

[c] Cochran's outlier

[d] Laboratory data not included in statistical analysis for all contamination levels

[e] FDA BAM and ISO 21257 reference method replicates were enumerated at 5 and 7 days (uninoculated test portions) of incubation at 25°C

Table 4. Total Yeast and Mold Log_{10} counts for frozen raw ground beef patties by 3M Petrifilm RYM Count Plate after 48 hours incubation at 28°C vs. FDA BAM and ISO 21527 reference methods[a]

	Uninoculated Control				Low				Medium				High			
	Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]	
Lab	A[b]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	<1.00	<1.00	<1.00	<1.00	2.18	1.48	2.23	1.60	3.49	3.38	3.49	3.32	4.64	4.66	4.32	4.54
2	<1.00	<1.00	<1.00	<1.00	2.38	2.57	2.43	2.83	3.68	3.62	3.49	3.45	4.64	4.68	4.53	4.52
3	<1.00	<1.00	<1.00	<1.00	2.30	2.45	2.41	2.32	3.66	3.30	3.63	3.53	4.68	4.40	4.82	4.81
4[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	<1.00	<1.00	<1.00	<1.00	1.70	2.04	1.70	2.00	3.56	3.51	3.30	3.41	4.53	4.53	4.46	4.64
6[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	<1.00	<1.00	<1.00	<1.00	2.18	2.26	1.80	1.82	3.46	3.61	3.36	3.61	4.65	4.54	4.43	4.49
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	<1.00	<1.00	<1.00	<1.00	2.30	2.26	2.28	2.23	3.59	3.54	3.51	3.45	4.66	4.75	4.57	4.79
10	<1.00	<1.00	<1.00	<1.00	2.20	2.15	2.11	2.00	3.60	3.45	3.45	3.43	4.79	4.60	4.66	4.40
11	<1.00	<1.00	<1.00	<1.00	2.48	1.70	2.40	1.90	3.56	3.64	3.49	3.54	4.73	4.66	4.49	4.59
12[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	<1.00	<1.00	<1.00	<1.00	2.52	2.18	2.00	1.67	3.41	3.41	3.52	3.48	4.80	4.92	4.67	4.77
14	<1.00	<1.00	<1.00	<1.00	2.23	1.60	2.34[c]	1.00[c]	3.60	3.59	3.57	3.70	4.71	4.67	4.65	4.62
15	<1.00	<1.00	<1.00	<1.00	2.34	2.20	2.15	2.23	3.49	3.41	3.38	3.23	4.72	4.73	4.61	4.66

RYM= 3M Petrifilm Rapid Yeast and Mold method.

FDA BAM= FDA BAM Chapter 18 method for enumeration of yeasts, molds and mycotoxins.

ISO 21527= ISO 21527:2008 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds – Part 1: Colony count technique in products with water activity greater than 0,95

[a] Log_{10} yeast and mold CFU/g;

[b] A and B= Indicated duplicate test portions;

[c] Cochran's outlier

[d] Laboratory data not included in statistical analysis for all contamination levels

[e] FDA BAM and ISO 21257 reference method replicates were enumerated at 5 and 7 days (uninoculated test portions) of incubation at 25°C

Table 5. Total Yeast and Mold Log_{10} counts for frozen raw ground beef patties by 3M Petrifilm RYM Count Plate after 60 hours incubation at 28°C vs. FDA BAM and ISO 21527 reference methods[a]

	Uninoculated Control				Low				Medium				High			
	Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]		Petrifilm RYM		FDA BAM/ ISO 21527[e]	
Lab	A[b]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	<1.00	<1.00	<1.00	<1.00	2.18	1.48	2.23	1.60	3.52	3.38	3.49	3.32	4.64	4.62	4.32	4.54
2	<1.00	<1.00	<1.00	<1.00	2.38	2.57	2.43	2.83	3.69	3.62	3.49	3.45	4.65	4.68	4.53	4.52
3	<1.00	<1.00	<1.00	<1.00	2.30	2.26	2.41	2.32	3.59	3.30	3.63	3.53	4.65	4.40	4.82	4.81
4[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	<1.00	<1.00	<1.00	<1.00	1.70	2.04	1.70	2.00	3.56	3.51	3.30	3.41	4.53	4.53	4.46	4.64
6[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
7	<1.00	<1.00	<1.00	<1.00	2.18	2.26	1.80	1.82	3.46	3.61	3.36	3.61	4.65	4.54	4.43	4.49
8[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
9	<1.00	<1.00	<1.00	<1.00	2.30	2.26	2.28	2.23	3.59	3.54	3.51	3.45	4.66	4.75	4.57	4.79
10	<1.00	<1.00	<1.00	<1.00	2.20	2.15	2.11	2.00	3.60	3.45	3.45	3.43	4.79	4.60	4.66	4.40
11	<1.00	<1.00	<1.00	<1.00	2.48	1.70	2.40	1.90	3.56	3.64	3.49	3.54	4.73	4.66	4.49	4.59
12[d]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
13	<1.00	<1.00	<1.00	<1.00	2.54	2.18	2.00	1.67	3.41	3.41	3.52	3.48	4.81	4.92	4.67	4.77
14	<1.00	<1.00	<1.00	<1.00	2.23	1.60	2.34[c]	1.00[c]	3.60	3.59	3.57	3.70	4.71	4.68	4.65	4.62
15	<1.00	<1.00	<1.00	<1.00	2.34	2.20	2.15	2.23	3.49	3.41	3.38	3.23	4.72	4.73	4.61	4.66

RYM= 3M Petrifilm Rapid Yeast and Mold method.

FDA BAM= FDA BAM Chapter 18 method for enumeration of yeasts, molds and mycotoxins.

ISO 21527 =ISO 21527:2008 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds – Part 1: Colony count technique in products with water activity greater than 0,95

[a] Log_{10} yeast and mold CFU/g;

[b] A and B= Indicated duplicate test portions;

[c] Cochran's outlier;

[d] Laboratory data not included in statistical analysis for all contamination levels

[e] FDA BAM and ISO 21257 reference method replicates were enumerated at 5 and 7 days (uninoculated test portions) of incubation at 25°C

Table 6. Total Yeast and Mold Log_{10} counts for raw almonds by 3M Petrifilm RYM Count Plate after 48 hours incubation at 25°C vs. FDA BAM and ISO 21527 reference methods[a]

	Uninoculated Control				Low				Medium				High			
	Petrifilm RYM		FDA BAM/ ISO 21527[h]		Petrifilm RYM		FDA BAM/ ISO 21527[h]		Petrifilm RYM		FDA BAM/ ISO 21527[h]		Petrifilm RYM		FDA BAM/ ISO 21527[h]	
Lab	A[b]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	<1.00	<1.00	1.00[f]	<1.00	1.30	1.30	1.78	1.85	1.60	1.30	2.28	1.95	1.78[e]	1.90[e]	2.04[d]	2.40[d]
2	1.30[f]	1.00[f]	1.30[f]	1.85[f]	1.78	1.60	2.04	1.70	1.30	1.90	2.11	2.34	1.95[e]	2.15[e]	2.64	2.84
3	<1.00	<1.00	<1.00	<1.00	1.30	1.30	1.60	1.60	2.34	2.28	2.38	2.18	3.56	3.11	3.30	3.11
4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	<1.00	<1.00	<1.00	<1.00	1.30	1.00	1.78	1.95	2.30	2.15	2.30	2.45	3.41	3.04	3.18	3.18
6	<1.00	<1.00	<1.00	<1.00	1.48	1.30	2.20	1.78	1.00[c]	2.00[c]	2.34	2.20	3.08	3.15	3.36	3.28
7	<1.00	<1.00	<1.00	<1.00	1.78	1.30	1.70	1.30	2.45	2.20	2.43	2.11	3.20	2.90	3.20	3.11
8	<1.00	<1.00	<1.00	<1.00	1.85	1.95	1.00	1.00	2.49	2.45	2.00	2.08	3.26	3.51	3.00	3.08
9	<1.00	<1.00	<1.00	<1.00	1.30	1.30	1.48	1.00	2.53	2.46	2.56	2.53	3.08	3.00	3.04	3.00
10	<1.00	<1.00	<1.00	<1.00	1.30	1.00	1.30	1.00	2.08	2.43	2.00	2.56	3.26	3.11	3.30	3.04
11	<1.00	<1.00	<1.00	<1.00	1.00	1.48	1.48	1.60	2.00	2.08	1.95	1.90	3.08	3.04	3.15	3.08
12	<1.00	<1.00	<1.00	<1.00	1.60	1.78	1.30	1.00	2.08	2.30	2.00	2.00	3.60	3.36	3.40	3.08
13	<1.00	<1.00	<1.00	<1.00	1.48	1.30	1.48	1.30	2.00	2.20	1.78	1.95	2.90	2.90	2.90	2.95
14	<1.00	<1.00	<1.00	<1.00	1.48	1.60	1.70	1.78	2.45	2.23	2.76	2.08	3.43	3.00	3.40	3.23
15	<1.00	<1.00	<1.00	<1.00	1.78	1.70	1.78	1.85	2.45	2.26	2.45	2.30	3.23	2.90	3.41	3.41

RYM= 3M Petrifilm Rapid Yeast and Mold method.

FDA BAM= FDA BAM Chapter 18 method for enumeration of yeasts, molds and mycotoxins.

ISO 21527 =ISO 21527:2008 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds – Part 2: Colony count technique in products with water activity less than or equal to 0,95

[a] Log_{10} yeast and mold CFU/g;

[b] A and B= Indicated duplicate test portions;

[c] Cochran's outlier;

[d] Single Grubbs' outliner;

[e] Double Grubbs' outliner;

[f] Data not included in statistical analysis for this contamination level

[g] Laboratory data not included in statistical analysis for all contamination levels

[h] FDA BAM and ISO 21257 reference method replicates were enumerated at 5 and 7 days (uninoculated test portions) of incubation at 25°C

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Table 7. Total Yeast and Mold Log_{10} counts for raw almonds by 3M Petrifilm RYM Count Plate after 60 hours incubation at 25°C vs. FDA BAM and ISO 21527 reference methods[a]

	Uninoculated Control				Low				Medium				High			
	Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]	
Lab	A[b]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	<1.00	<1.00	1.00[e]	<1.00	1.48	1.78	1.78	1.85	1.90	1.60	2.28	1.95	2.00[d]	2.20[d]	2.04[c]	2.40[c]
2	1.30[e]	1.00[e]	1.30[e]	1.85[e]	1.90	1.60	2.04	1.70	1.48	2.15	2.11	2.34	2.28[d]	2.38[d]	2.64	2.84
3	<1.00	<1.00	<1.00	<1.00	1.30	1.30	1.60	1.60	2.38	2.28	2.38	2.18	3.56	3.11	3.30	3.11
4[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	<1.00	<1.00	<1.00	<1.00	1.60	1.70	1.78	1.95	2.30	2.15	2.30	2.45	3.41	3.04	3.18	3.18
6	<1.00	<1.00	<1.00	<1.00	1.78	1.00	2.20	1.78	2.30	1.78	2.34	2.20	3.08	3.08	3.36	3.28
7	<1.00	<1.00	<1.00	<1.00	1.78	1.30	1.70	1.30	2.45	2.20	2.43	2.11	3.23	3.08	3.20	3.11
8	<1.00	<1.00	<1.00	<1.00	1.85	1.95	1.00	1.00	2.45	2.49	2.00	2.08	3.26	3.51	3.00	3.08
9	<1.00	<1.00	<1.00	<1.00	1.30	1.30	1.48	1.00	2.56	2.48	2.56	2.53	3.00	3.04	3.04	3.00
10	<1.00	<1.00	<1.00	<1.00	1.30	1.00	1.30	1.00	2.08	2.43	2.00	2.56	3.26	3.11	3.30	3.04
11	<1.00	<1.00	<1.00	<1.00	1.00	1.48	1.48	1.60	2.00	2.08	1.95	1.90	3.08	3.04	3.15	3.08
12	<1.00	<1.00	<1.00	<1.00	1.78	1.90	1.30	1.00	2.08	2.30	2.00	2.00	3.65	3.36	3.40	3.08
13	<1.00	<1.00	<1.00	<1.00	1.48	1.30	1.48	1.30	2.20	2.00	1.78	1.95	2.90	2.90	2.90	2.95
14	<1.00	<1.00	<1.00	<1.00	1.48	1.60	1.70	1.78	2.45	2.23	2.76	2.08	3.45	3.00	3.40	3.23
15	<1.00	<1.00	<1.00	<1.00	1.78	1.70	1.78	1.85	2.26	2.45	2.45	2.30	3.23	2.90	3.41	3.41

RYM= 3M Petrifilm Rapid Yeast and Mold method.

FDA BAM= FDA BAM Chapter 18 method for enumeration of yeasts, molds and mycotoxins.

ISO 21527= ISO 21527:2008 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds - Part 2: Colony count technique in products with water activity less than or equal to 0,95

[a] Log_{10} yeast and mold CFU/g;

[b] A and B= Indicated duplicate test portions;

[c] Single Grubbs' outliner;

[d] Double Grubbs' outliner;

[e] Data not included in statistical analysis for this contamination level

[f] Laboratory data not included in statistical analysis for all contamination levels

[g] FDA BAM and ISO 21257 reference method replicates were enumerated at 5 and 7 days (uninoculated test portions) of incubation at 25°C

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Table 8. Total Yeast and Mold Log_{10} counts for raw almonds by 3M Petrifilm RYM Count Plate after 48 hours incubation at 28°C vs. FDA BAM and ISO 21527 reference methods[a]

	Uninoculated Control				Low				Medium				High			
	Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]	
Lab	A[b]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	1.00[e]	1.00[e]	1.00[e]	<1.00	1.30	1.48	1.78	1.85	1.78	1.90	2.28	1.95	1.90[d]	2.20[d]	2.04[c]	2.40[c]
2	1.30[e]	1.30[e]	1.30[e]	1.85[e]	1.78	1.60	2.04	1.70	1.78	1.78	2.11	2.34	2.11[d]	2.11[d]	2.64	2.84
3	<1.00	<1.00	<1.00	<1.00	1.78	1.30	1.60	1.60	2.34	2.30	2.38	2.18	3.57	3.15	3.30	3.11
4[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	<1.00	<1.00	<1.00	<1.00	1.60	1.78	1.78	1.95	2.30	2.00	2.30	2.45	3.34	3.15	3.18	3.18
6	<1.00	<1.00	<1.00	<1.00	1.78	1.30	2.20	1.78	1.78	1.30	2.34	2.20	3.04	3.11	3.36	3.28
7	<1.00	<1.00	<1.00	<1.00	1.60	1.48	1.70	1.30	2.36	2.20	2.43	2.11	3.18	3.04	3.20	3.11
8	<1.00	<1.00	<1.00	<1.00	1.85	1.90	1.00	1.00	2.43	2.45	2.00	2.08	3.20	3.40	3.00	3.08
9	<1.00	<1.00	<1.00	<1.00	1.30	1.30	1.48	1.00	2.53	2.45	2.56	2.53	3.08	3.00	3.04	3.00
10	<1.00	<1.00	<1.00	<1.00	1.30	1.30	1.30	1.00	2.11	2.38	2.00	2.56	3.26	3.18	3.30	3.04
11	<1.00	<1.00	<1.00	<1.00	1.60	1.30	1.48	1.60	2.15	2.30	1.95	1.90	3.15	3.15	3.15	3.08
12	<1.00	<1.00	<1.00	<1.00	1.70	1.85	1.30	1.00	2.28	2.26	2.00	2.00	3.70	3.34	3.40	3.08
13	<1.00	<1.00	<1.00	<1.00	1.60	1.60	1.48	1.30	2.15	2.28	1.78	1.95	2.78	2.90	2.90	2.95
14	<1.00	<1.00	<1.00	<1.00	1.60	1.70	1.70	1.78	2.53	2.08	2.76	2.08	3.49	3.08	3.40	3.23
15	<1.00	<1.00	<1.00	<1.00	1.78	1.78	1.78	1.85	2.11	2.45	2.45	2.30	3.08	2.70	3.41	3.41

RYM= 3M Petrifilm Rapid Yeast and Mold method.

FDA BAM= FDA BAM Chapter 18 method for enumeration of yeasts, molds and mycotoxins.

ISO 21527= ISO 21527:2008 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds –Part 2: Colony count technique in products with water activity less than or equal to 0,95

[a] Log_{10} yeast and mold CFU/g;

[b] A and B= Indicated duplicate test portions;

[c] Single Grubbs' outliner;

[d] Double Grubbs' outliner;

[e] Data not included in statistical analysis for this contamination level

[f] Laboratory data not included in statistical analysis for all contamination levels

[g] FDA BAM and ISO 21257 reference method replicates were enumerated at 5 and 7 days (uninoculated test portions) of incubation at 25°C

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Table 9. Total Yeast and Mold Log_{10} counts for raw almonds by 3M Petrifilm RYM Count Plate after 60 hours incubation at 28°C vs. FDA BAM and ISO 21527 reference methods[a]

	Uninoculated Control				Low				Medium				High			
	Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]		Petrifilm RYM		FDA BAM/ ISO 21527[g]	
Lab	A[b]	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
1	1.00[e]	1.00[e]	1.00[e]	<1.00	1.60	1.48	1.78	1.85	1.90	1.95	2.28	1.95	1.90[d]	2.26[d]	2.04[c]	2.40[c]
2	1.30[e]	1.30[e]	1.30[e]	1.85[e]	1.85	1.60	2.04	1.70	1.78	2.11	2.11	2.34	2.28[d]	2.34[d]	2.64	2.84
3	<1.00	<1.00	<1.00	<1.00	1.78	1.30	1.60	1.60	2.38	2.30	2.38	2.18	3.57	3.15	3.30	3.11
4[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
5	<1.00	<1.00	<1.00	<1.00	1.60	1.78	1.78	1.95	2.30	2.00	2.30	2.45	3.34	3.15	3.18	3.18
6	<1.00	<1.00	<1.00	<1.00	1.78	1.30	2.20	1.78	1.95	1.60	2.34	2.20	3.04	3.15	3.36	3.28
7	<1.00	<1.00	<1.00	<1.00	1.70	1.48	1.70	1.30	2.36	2.20	2.43	2.11	3.18	3.04	3.20	3.11
8	<1.00	<1.00	<1.00	<1.00	1.85	1.90	1.00	1.00	2.43	2.45	2.00	2.08	3.20	3.40	3.00	3.08
9	<1.00	<1.00	<1.00	<1.00	1.48	1.30	1.48	1.00	2.54	2.51	2.56	2.53	3.00	3.04	3.04	3.00
10	<1.00	<1.00	<1.00	<1.00	1.30	1.30	1.30	1.00	2.11	2.38	2.00	2.56	3.26	3.18	3.30	3.04
11	<1.00	<1.00	<1.00	<1.00	1.60	1.30	1.48	1.60	2.15	2.30	1.95	1.90	3.15	3.15	3.15	3.08
12	<1.00	<1.00	<1.00	<1.00	1.70	1.90	1.30	1.00	2.28	2.26	2.00	2.00	3.70	3.34	3.40	3.08
13	<1.00	<1.00	<1.00	<1.00	1.60	1.60	1.48	1.30	2.15	2.28	1.78	1.95	2.78	2.90	2.90	2.95
14	<1.00	<1.00	<1.00	<1.00	1.60	1.70	1.70	1.78	2.53	2.08	2.76	2.08	3.51	3.08	3.40	3.23
15	<1.00	<1.00	<1.00	<1.00	1.78	1.78	1.78	1.85	2.11	2.45	2.45	2.30	3.08	2.70	3.41	3.41

RYM= 3M Petrifilm Rapid Yeast and Mold method.

FDA BAM= FDA BAM Chapter 18 method for enumeration of yeasts, molds and mycotoxins.

ISO 21527 =ISO 21527:2008 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration for yeast and molds –Part 2: Colony count technique in products with water activity less than or equal to 0,95

[a] Log_{10} yeast and mold CFU/g;

[b] A and B= Indicated duplicate test portions;

[c] Single Grubbs' outliner;

[d] Double Grubbs' outliner;

[e] Data not included in statistical analysis for this contamination level

[f] Laboratory data not included in statistical analysis for all contamination levels

[g] FDA BAM and ISO 21257 reference method replicates were enumerated at 5 and 7 days (uninoculated test portions) of incubation at 25°C

Table 2014.1. Interlaboratory study results of 3M Petrifilm RYM vs. the FDA BAM and ISO 21527 methods For Frozen Raw Ground Beef Patties																		
3M Petrifilm RYM Method								FDA BAM/ISO 21527 Methods[a]							p-value[d]	Difference of Means[e]	Reverse Transformed Mean Difference [f]
Matrix	Lot	N[b]	Mean[c]	s_r		s_R		Lot	N[b]	Mean[c]	s_r		s_R				
Frozen Raw Ground Beef Patties 25°C 48 hours	Control	11(0)	<1.00	-		-		Control	11(0)	<1.00	-		-		-	-	-
	Low	11(0)	2.12	0.41		0.41		Low	11(1)	2.07	0.36		0.38		0.5323	0.05	14.34
	Medium	11(0)	3.52	0.10		0.10		Medium	11(0)	3.47	0.09		0.11		0.1637	0.05	360.10
	High	11(0)	4.65	0.13		0.14		High	11(0)	4.59	0.10		0.14		0.2266	0.06	5763.84
Frozen Raw Ground Beef Patties 25°C 60 hours	Control	11(0)	<1.00	-		-		Control	11(0)	<1.00	-		-		-	-	-
	Low	11(0)	2.14	0.36[g]		0.37		Low	11(1)	2.07	0.36		0.38		0.3773	0.07	20.55
	Medium	11(0)	3.52	0.10		0.10		Medium	11(0)	3.47	0.09		0.11		0.1573	0.05	360.10
	High	11(0)	4.65	0.14		0.15		High	11(0)	4.59	0.10		0.14		0.1750	0.06	5763.84
Frozen Raw Ground Beef Patties 28°C 48 hours	Control	11(0)	<1.00	-		-		Control	11(0)	<1.00	-		-		-	-	-
	Low	11(0)	2.17	0.29[g]		0.30		Low	11(1)	2.07	0.36		0.38		0.1391	0.10	30.42
	Medium	11(0)	3.53	0.10		0.10		Medium	11(0)	3.47	0.09		0.11		0.0824	0.06	437.23
	High	11(0)	4.67	0.08[g]		0.11		High	11(0)	4.59	0.10		0.14		0.0966	0.08	7869.00
Frozen Raw Ground Beef Patties 28°C 60 hours	Control	11(0)	<1.00	-		-		Control	11(0)	<1.00	-		-		-	-	-
	Low	11(0)	2.16	0.29[g]		0.29		Low	11(1)	2.07	0.36		0.38		0.1843	0.09	27.05
	Medium	11(0)	3.53	0.09		0.10		Medium	11(0)	3.47	0.09		0.11		0.1095	0.06	437.23
	High	11(0)	4.67	0.08[g]		0.11		High	11(0)	4.59	0.10		0.14		0.1088	0.08	7869.00

[a] Samples were analyzed by a harmonized FDA BAM Chapter 18 and ISO 21527 methods using 0.1% peptone as the sample diluent

[b] Number of laboratories that reported complete results. Outliers are presented in parentheses.

[c] Log_{10} yeast and mold CFU/g.

[d] Significant difference ($p<0.05$)

[e] Significant difference if absolute value is >0.5

[f] Results presented as CFU/g

[g] Results indicate that the candidate method is more repeatable then the reference methods

s_r – Repeatability;

s_R – Reproducibility;

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Table 2014.2. Interlaboratory study results of 3M Petrifilm RYM vs. the FDA BAM and ISO 21527 methods for Raw Almonds																		
3M Petrifilm RYM Method								FDA BAM/ISO 21527 Methods[a]							p-value[d]	Difference of Means[e]	Reverse Transformed Mean Difference [f]
Matrix	Lot	N[b]	Mean[c]	s_r		s_R		Lot	N[b]	Mean[c]	s_r		s_R				
Raw Almonds 25°C 48 hours	Control	12(0)	<1.00	-		-		Control	12(0)	<1.00	-		-		-	-	-
	Low	14(0)	1.45	0.17[g]		0.26		Low	14(0)	1.55	0.19		0.34		0.4165	0.10	-7.30
	Medium	14(1)	2.12	0.26		0.39		Medium	14(0)	2.21	0.20		0.24		0.3322	0.09	-30.36
	High	14(2)	3.00	0.18		0.49		High	14(1)	3.08	0.12		0.31		0.2833	0.08	-202.26
Raw Almonds 25°C 60 hours	Control	12(0)	<1.00	-		-		Control	12(0)	<1.00	-		-		-	-	-
	Low	14(0)	1.53	0.23		0.28		Low	14(0)	1.55	0.19		0.34		0.8391	0.02	-1.60
	Medium	14(0)	2.20	0.21		0.27		Medium	14(0)	2.21	0.20		0.24		0.7789	0.01	-3.69
	High	14(2)	3.04	0.18		0.41		High	14(1)	3.08	0.12		0.31		0.5418	0.04	-105.79
Raw Almonds 28°C 48 hours	Control	12(0)	<1.00	-		-		Control	12(0)	<1.00	-		-		-	-	-
	Low	14(0)	1.58	0.16[g]		0.21		Low	14(0)	1.55	0.19		0.34		0.7381	0.03	2.54
	Medium	14(0)	2.17	0.17[g]		0.29		Medium	14(0)	2.21	0.20		0.24		0.6139	0.04	-11.73
	High	14(2)	3.01	0.17		0.45		High	14(1)	3.08	0.12		0.31		0.3904	0.07	-178.97
Raw Almonds 28°C 60 hours	Control	12(0)	<1.00	-		-		Control	12(0)	<1.00	-		-		-	-	-
	Low	14(0)	1.60	0.17[g]		0.20		Low	14(0)	1.55	0.19		0.34		0.5474	0.05	4.33
	Medium	14(0)	2.21	0.17[g]		0.23		Medium	14(0)	2.21	0.20		0.24		0.9483	0.00	0.00
	High	14(2)	3.03	0.18		0.42		High	14(1)	3.08	0.12		0.31		0.4687	0.05	-130.75

[a] Samples were analyzed by a harmonized FDA BAM Chapter 18 and ISO 21527 methods using 0.1% peptone as the sample diluent

[b] Number of laboratories that reported complete results. Outliers are presented in parentheses.

[c] Log_{10} yeast and mold CFU/g.

[d] Significant difference ($p<0.05$)

[e] A mean difference absolute value of greater than 0.5 indicates a statistical significant difference between methods

[f] Results presented as CFU/g

[g] Results indicate that the candidate method is more repeatable then the reference methodss_r – Repeatability; s_R – Reproducibility;

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Table 2014.3: Results of Aerobic Plate Count for Collaborating Laboratories

Lab	Frozen Raw Ground Beef (CFU/g)	Raw Almonds (CFU/g)
1	3.8×10^2	6.0×10^1
2	1.1×10^3	6.0×10^2
3	<10	3.0×10^1
4	Not Reported	Not Reported
5	2.8×10^3	2.8×10^1
6	8.0×10^1	2.2×10^1
7	9.1×10^2	1.6×10^2
8	Not Reported	Not Reported
9	9.0×10^2	2.0×10^2
10	1.3×10^3	4.0×10^2
11	>2500	1.0×10^1
12	Not Reported	7.0×10^1
13	9.5×10^1	1.0×10^1
14	7.3×10^2	2.3×10^2
15	3.7×10^2	8.0×10^1

Figure 1: 3M Petrifilm RYM Count Plate vs. FDA BAM/ISO 21527 Results for Frozen Raw Ground Beef Patties Incubated at 25°C and Enumerated at 48 and 60 Hours

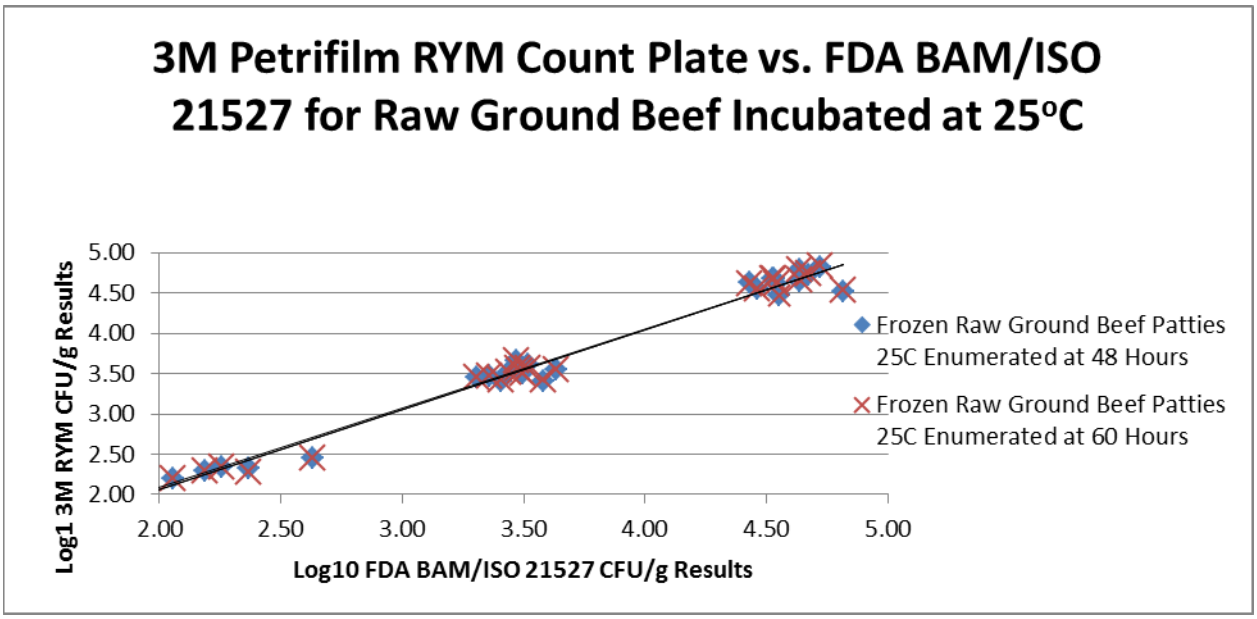


Figure 2: 3M Petrifilm RYM Count Plate vs. FDA BAM/ISO 21527 Results for Frozen Raw Ground Beef Patties Incubated at 28°C and Enumerated at 48 and 60 Hours

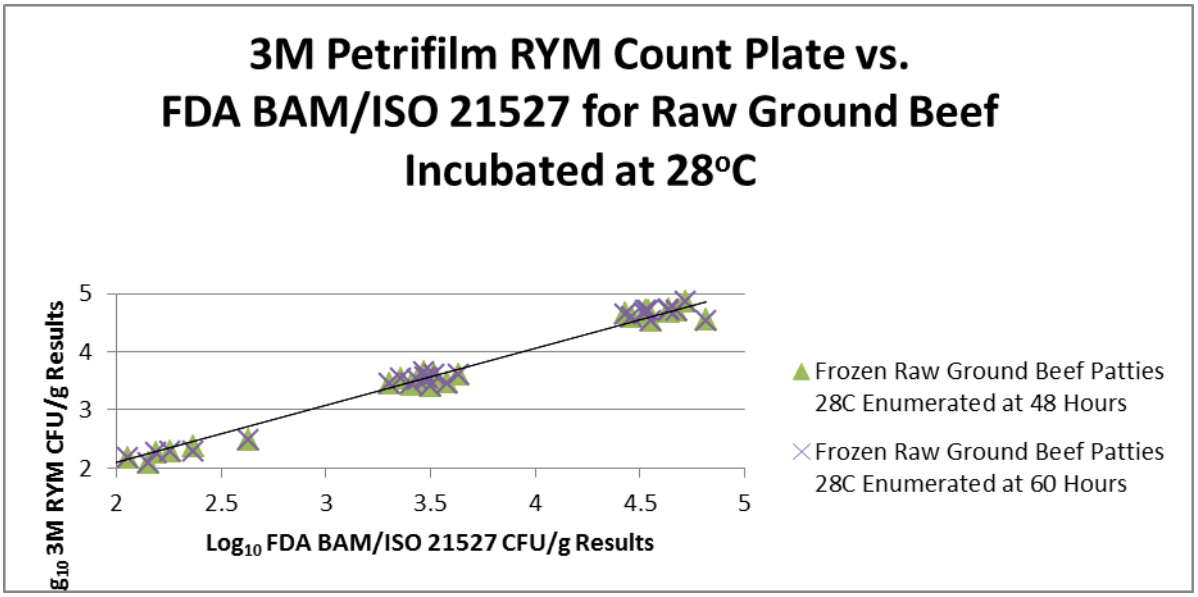
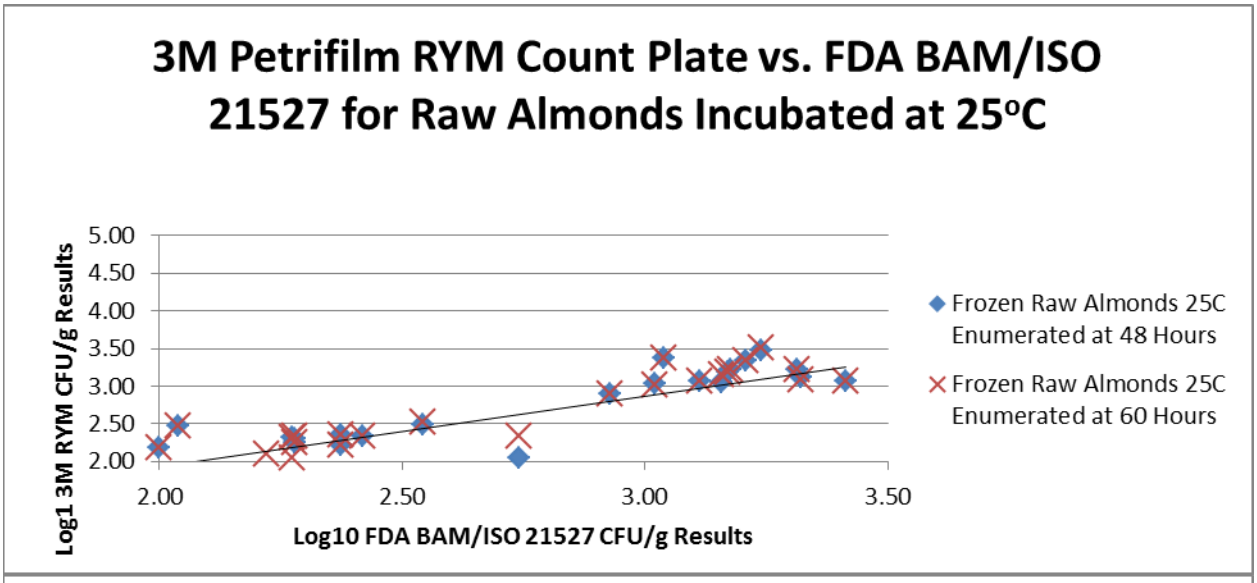


Figure 3: 3M Petrifilm RYM Count Plate vs. FDA BAM/ISO 21527 Results for Raw Almonds Incubated at 25°C and Enumerated at 48 and 60 Hours



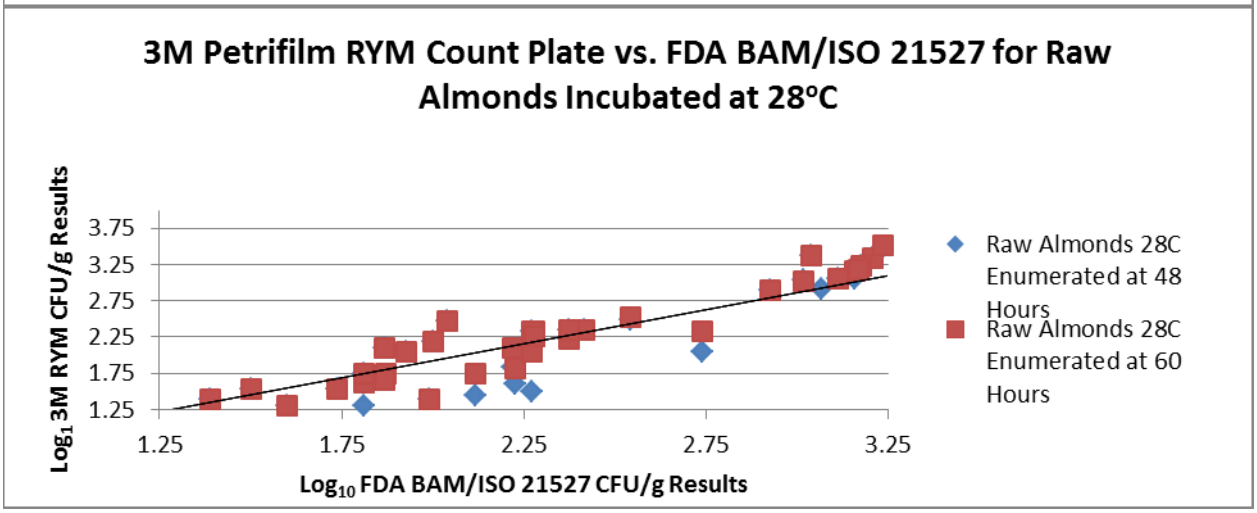
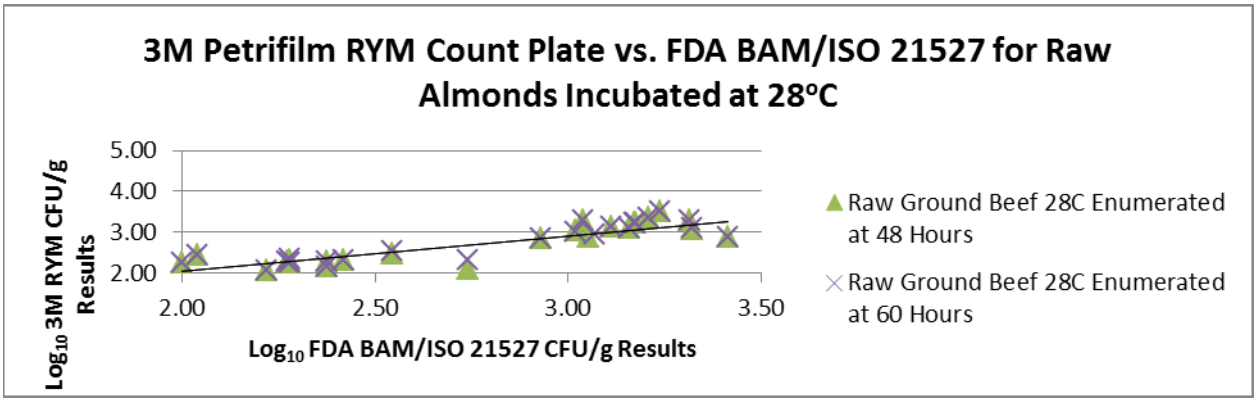


Figure 4: 3M Petrifilm RYM Count Plate vs. FDA BAM/ISO 21527 Results for Raw Almonds Incubated at 28°C and Enumerated at 48 and 60 Hours



AOAC Official Method 2014.06
***Listeria* species in Selected Foods and Environmental Surfaces**
3M™ Molecular Detection Assay (MDA) *Listeria* Method
First Action 2014

[Applicable to detection of *Listeria* species in selected foods, including beef hot dogs (25 g), deli turkey (25 g), cold smoked salmon (25 g), full-fat cottage cheese (25 g), and two environmental surfaces: sealed concrete (sponge in 100 mL and sponge in 225 mL enrichment volume) and stainless steel (swab in 10 mL and sponge in 225 mL enrichment volume) enriched in prewarmed Demi-Fraser (DF) broth base.]

See Table **2014.06A** for a summary of results of the interlaboratory study supporting acceptance of the method.

See Appendix available on the *J. AOAC Int*. website for supplementary materials for detailed results of the interlaboratory study (http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

A. Principle

The 3M™ Molecular Detection Assay (MDA) *Listeria* is intended for use with the 3M Molecular Detection System for the rapid and specific detection of *Listeria* spp. in selected foods and environmental surfaces. The 3M MDA uses loop-mediated isothermal amplification to rapidly amplify nucleic acid sequences with high specificity and sensitivity, combined with bioluminescence to detect the amplification. Presumptive positive results are reported in real-time, while negative results are displayed after the assay is completed. Samples are enriched in prewarmed DF broth base, which does not contain ferric ammonium citrate (FAC).

B. Apparatus and Reagents

Items (**a**) and (**h**)-(**o**) are available from 3M Food Safety (St. Paul, MN, USA). Items (**b**)-(**g**) are available as the 3M MDA *Listeria* kit from 3M Food Safety.

(**a**) *3M™ Molecular Detection Instrument.*

(**b**) *3M MDA Listeria reagent tubes.*--Twelve strips of eight tubes.

(**c**) *Lysis solution (LS) tubes.*–Twelve strips of eight tubes.

(**d**) *Extra caps.*–Twelve strips of eight caps.

(**e**) *Negative control (NC).*--One vial (2 mL).

(**f**) *Reagent control (RC).*–Two pouches. Each pouch contains eight reagent tubes.

(**g**) *Quick Start Guide.*

(**h**) *3M™ Molecular Detection Speed Loader Tray.*

(**i**) *3M™ Molecular Detection Chill Block Tray and Chill Block Insert.*

(**j**) *3M™ Molecular Detection Heat Block Insert.*

(**k**) *3M™ Molecular Detection Cap/Decap Tool-Reagent (for reagent tubes).*

(**l**) *3M™ Molecular Detection Cap/Decap Tool-Lysis (for lysis tubes).*

(**m**) *Empty lysis tube rack.*

(**n**) *Empty reagent tube rack.*

(**o**) *DF broth base.*–Formulation equivalent to ISO 11290-1:1996.

(**p**) *Disposable pipet.*–Capable of 20 µL.

(**q**) *Multichannel (8-channel) pipet.*--Capable of 20 µL.

(**r**) *Sterile filter tip pipet tips.*--Capable of 20 µL.

(**s**) *Filter Stomacher® bags.*–Seward or equivalent.

(**t**) *Stomacher.*–Seward or equivalent.

(**u**) *Thermometer.*–Calibrated range to include 100 ± 1°C.

(**v**) *Dry double block heater unit or water bath.*–Capable of maintaining 100 ± 1°C.

(**w**) *Incubators.*–Capable of maintaining 37 ± 1°C.

(**x**) *Freezer.*–Capable of maintaining -10 to -20°C, for storing the 3M Molecular Detection Chill Block Tray.

(**y**) *Refrigerator.*–Capable of maintaining 2-8°C, for storing the 3M MDA.

(**z**) *Computer.*–Compatible with the 3M Molecular Detection Instrument.

C. General Instructions

(**a**) Store the 3M MDA *Listeria* at 2-8°C. Do not freeze. Keep kit away from light during storage. After opening the kit, check that the foil pouch is undamaged. If the pouch is damaged, do not use. After opening, unused reagent tubes should always be stored in the resealable pouch with the desiccant inside to maintain stability of the lyophilized reagents. Store resealed pouches at 2-8°C for no longer than 1 month. Do not use 3M MDA *Listeria* past the expiration date.

(**b**) The 3M Molecular Detection Instrument is intended for use with samples that have undergone heat treatment during the assay lysis step, which is designed to destroy organisms present in the sample. Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should *not* be inserted into the 3M Molecular Detection Instrument.

(**c**) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

D. Safety Precautions

Periodically decontaminate laboratory benches and equipment (pipets, cap/decap tools, etc.) with a 1-5% (v/v in water) household bleach solution or DNA removal solution.

Listeria monocytogenes is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these groups of concern avoid handling this organism. After use, the enrichment medium and the 3M MDA *Listeria* tubes can potentially contain pathogenic materials. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet (MSDS) for additional information and local regulations for disposal.

Ethanol used in the method is flammable and caution should be used. Consult MSDS for additional information.

E. Sample Enrichment

(**a**) Prewarm DF broth base without FAC to 37 ± 1°C.

(**b**) Aseptically combine the enrichment medium and sample following the procedures in Table **2014.06B**. For all meat and highly particulate samples, the use of filter bags is recommended. Homogenize thoroughly (Stomacher, blender) for 2 ± 0.5 min. Incubate at 37 ± 1°C.

F. Preparation of the 3M Molecular Detection Speed Loader Tray

(**a**) Wet a cloth or paper towel with a 1-5% (v/v in water) household bleach solution and wipe the 3M Molecular Detection Speed Loader Tray.

(**b**) Rinse the 3M Molecular Detection Speed Loader Tray with water.

(**c**) Use a disposable towel to wipe the 3M Molecular Detection Speed Loader Tray dry.

(**d**) Ensure the 3M Molecular Detection Speed Loader Tray is dry before use.

G. Preparation of the 3M Molecular Detection Chill Block Insert

Before using the 3M Molecular Detection Chill Block Insert, ensure it has been stored on the 3M Molecular Detection Chill Block Tray in the freezer (-10 to -20°C) for a minimum of 2 h before use. When removing the 3M Molecular Detection Chill Block Insert from the freezer for use, remove it and the 3M Molecular Detection Chill Block Tray together. Use the 3M Molecular Detection Chill Block Insert/3M Molecular Detection Chill Block Tray within 20 min.

H. Preparation of the 3M Molecular Detection Heat Block Insert

Place the 3M Molecular Detection Heat Block Insert in a dry double block heater unit. Turn on the dry block heater unit and set the temperature to allow the 3M Molecular Detection Heat Block Insert to reach and maintain a temperature of 100 ± 1°C.

Note: Depending on the heater unit, allow approximately 30-50 min for the 3M Molecular Detection Heat Block Insert to reach temperature. Using a calibrated thermometer, verify that the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C.

I. Preparation of the 3M Molecular Detection Instrument

(**a**) Launch the 3M Molecular Detection Software and log in.

(**b**) Turn on the 3M Molecular Detection Instrument.

(**c**) Create or edit a run with data for each sample. Refer to the 3M Molecular Detection System User Manual for details.

Note: The 3M Molecular Detection Instrument must reach and maintain temperature of 60°C before inserting the 3M Molecular Detection Speed Loader Tray with reaction tubes. This heating step takes approximately 20 min and is indicated by an orange light on the instrument's status bar. When the instrument is ready to start a run, the status bar will turn green.

J. Lysis

(**a**) Allow the LS tubes to warm up to room temperature (20-25°C) by setting the rack on the laboratory bench for 2 h. *Alternatives to equilibrate the LS tubes to room temperature are to incubate the LS tubes in a 37 ± 1°C incubator for 1 h or at room temperature overnight (16-18 h).*

(**b**) Remove the enrichment broth from the incubator and gently agitate the contents.

(**c**) One LS tube is required for each sample and the NC sample.

(*1*) LS tube strips can be cut to desired LS tube number. Select the number of individual LS tubes or 8-tube strips needed. Place the LS tubes in an empty rack.

(*2*) To avoid cross-contamination, decap one LS tube strip at a time and use a new pipet tip for each transfer step.

(**d**) Transfer enriched sample to LS tubes as described below:

Note: Transfer each enriched sample into individual LS tubes first. Transfer the NC last.

(*1*) Use the 3M Molecular Detection Cap/Decap Tool-Lysis to decap one LS tube strip, one strip at a time. Set the tool with cap attached aside on a clean surface.

(*2*) Transfer 20 µL of sample into an LS tube.

(*3*) Repeat step (**d**)(*2*) until each individual sample has been added to a corresponding LS tube in the strip.

(*4*) Use the 3M Molecular Detection Cap/Decap Tool-Lysis to recap the LS tube strip. Use the rounded side of the tool to apply pressure in a back-and-forth motion ensuring that the cap is tightly applied. *See* Figure **2014.06A**.

(*5*) Repeat steps (**d**)(*1*)-(**d**)(*4*) as needed, for the number of samples to be tested.

(*6*) When all samples have been transferred, then transfer 20 µL NC into an LS tube. Use the 3M Molecular Detection Cap/Decap Tool-Lysis tool to recap the LS tube.

(*7*) Cover the rack of LS tubes with the rack lid and firmly invert 3-5 times to mix. *Suspension has to flow freely inside the tube.*

(**e**) Verify that the temperature of the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C. Place the rack of LS tubes in the 3M Molecular Detection Heat Block Insert and heat for 15 ± 1 min. *An alternative to using dry heat for the lysis step is to use a water bath at 100 ± 1°C. Ensure that sufficient water is used to cover up to the liquid level in the LS tubes. Place the rack of LS tubes in the water bath at 100 ± 1°C and heat for 15 ± 1 min.* Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should *not* be inserted into the 3M Molecular Detection Instrument.

(**f**) Remove the rack of LS tubes from the heating block and allow to cool in the 3M Molecular Detection Chill Block Insert for 10 ± 1 min. *The LS solution may freeze when processing less than 48 LS tubes. Freezing of the LS solution will not affect the test. If freezing is observed, allow the LS tubes to thaw for 5 min before mixing. Remove the rack lid during incubation on the 3M Molecular Detection Chill Block Insert.*

(**g**) Remove the rack of LS tubes from the 3M Molecular Detection Chill Block Insert/3M Molecular Detection Chill Block Tray system. Replace the lid on the rack of LS tubes and firmly invert 3-5 times to mix. *Suspension has to flow freely inside the tube.*

(**h**) Firmly tap the lysis tubes rack on the laboratory bench 3-5 times.

(**i**) Place the rack on the laboratory bench and let sit undisturbed for 5-10 min to allow the resin to settle. *Do not mix or disturb the resin at the bottom of the tube. See* Figure **2014.06B**.

K. Amplification

(**a**) One reagent tube is required for each sample and the NC.

(*1*) Reagent tube strips can be cut to desired tube number. Select the number of individual reagent tubes or 8-tube strips needed.

(*2*) Place reagent tubes in an empty rack.

(*3*) Avoid disturbing the reagent pellets from the bottom of the tubes.

(**b**) Select one RC tube and place in rack.

(**c**) To avoid cross-contamination, decap one reagent tube strip at a time and use a new pipet tip for each transfer step.

(**d**) Transfer lysate to reagent tubes and RC tube as described below:

Note: Transfer each sample lysate into individual reagent tubes first followed by the NC. Hydrate the RC tube last.

Warning: Care must be taken when pipetting LS, as carry-over of the resin may interfere with amplification.

(*1*) Use the 3M Molecular Detection Cap/Decap Tool-Reagent to decap the reagent tubes, one reagent tube strip at a time. Discard cap.

(*2*) Transfer 20 µL of sample lysate from the upper portion of the fluid in the LS tube into corresponding reagent tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down 5 times.

(*3*) Repeat step (**d**)(*2*) until individual sample lysate has been added to a corresponding reagent tube in the strip.

(*4*) Cover the reagent tubes with the provided extra cap and use the rounded side of the 3M Molecular Detection Cap/Decap Tool-Reagent to apply pressure in a back and forth motion ensuring that the cap is tightly applied.

(*5*) Repeat steps (**d**)(*1*)-(**d**)(*4*) as needed, for the number of samples to be tested.

(*6*) When all sample lysates have been transferred, repeat steps (**d**)(*1*)-(**d**)(*4*) to transfer 20 µL NC lysate into a reagent tube.

(*7*) Transfer 20 µL NC lysate into an RC tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down 5 times.

(**e**) Load capped tubes into a clean and decontaminated 3M Molecular Detection Speed Loader Tray. Close and latch the 3M Molecular Detection Speed Loader Tray lid (Figure **2014.06C**).

(**f**) Review and confirm the configured run in the 3M Molecular Detection Software.

(**g**) Click the Start button in the software and select instrument for use. The selected instrument's lid automatically opens.

(**h**) Place the 3M Molecular Detection Speed Loader Tray into the 3M Molecular Detection Instrument and close the lid to start the assay. Results are provided within 75 min, although positives may be detected sooner.

(**i**) After the assay is complete, remove the 3M Molecular Detection Speed Loader Tray from the 3M Molecular Detection Instrument and dispose of the tubes by soaking in a 1-5% (v/v in water) household bleach solution for 1 h and away from the assay preparation area.

Notice: To minimize the risk of false positives due to cross-contamination, never open reagent tubes containing amplified DNA. This includes RC, reagent, and Matrix Control tubes. Always dispose of sealed reagent tubes by soaking in a 1-5% (v/v in water) household bleach solution for 1 h and away from the assay preparation area.

L. Results and Interpretation

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A positive or negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real-time while Negative and Inspect results will be displayed after the run is completed. Presumptive positive results should be confirmed using one's preferred method or as specified by the FDA/BAM, USDA/FSIS-MLG, AOAC *Official Method*^{SM} **993.12**, or ISO 11290 methods starting from the 3M primary enrichment, followed by transfer to a secondary enrichment or direct plating onto media through confirmation of isolates using appropriate biochemical and serological methods.

Note: Even a negative sample will not give a zero reading as the system and 3M Molecular Assay *Listeria* amplification reagents have a "background" relative light unit (RLU).

In the rare event of any unusual light output, the algorithm labels this as "Inspect." 3M recommends the user to repeat the assay for any Inspect samples. If the result continues to be Inspect, proceed to confirmation test using one's preferred method or as specified by local regulations.

Reference: *J. AOAC Int*. (future issue)

Posted: January 29, 2015

Table 2014.06A. Summary of results for the detection of *Listeria* in full-fat cottage cheese (25 g)

Method[a]	3M™ MDA™ *Listeria*		
Inoculation level	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	1/132	67/132	132/132
Candidate presumptive POD (CP)	0.01 (0.00, 0.04)	0.51 (0.42, 0.60)	1.00 (0.97, 1.00)
s_r[b]	0.09 (0.08, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.04)	0.00 (0.00, 0.17)	0.00 (0.00, 0.16)
s_R[d]	0.09 (0.08, 0.10)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P-value[e]	0.4338	0.8931	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/132	64/132	132/132
Candidate confirmed POD (CC)	0.00 (0.00, 0.03)	0.48 (0.40, 0.57)	1.00 (0.97, 1.00)
s_r	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.16)	0.00 (0.00, 0.15)	0.00 (0.00, 0.16)
s_R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P-value	1.0000	0.8762	1.0000
Positive reference samples/total No. of samples analyzed	0/132	73/132	132/132
Reference POD	0.00 (0.00, 0.03)	0.55 (0.47, 0.64)	1.00 (0.97, 1.00)
s_r	0.00 (0.00, 0.16)	0.50 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.16)	0.00 (0.00, 0.18)	0.00 (0.00, 0.16)
s_R	0.00 (0.00, 0.23)	0.50 (0.45, 0.52)	0.00 (0.00, 0.23)
P-value	1.0000	0.6678	1.0000
dLPOD (candidate vs reference)[f]	0.00 (-0.03, 0.03)	-0.07 (-0.19, 0.06)	0.00 (-0.03, 0.03)
dLPOD (candidate presumptive vs candidate confirmed)[f]	0.01 (-0.02, 0.04)	0.01 (-0.12, 0.13)	0.00 (-0.03, 0.03)

[a] Results include 95% confidence intervals.

[b] s_r = Repeatability standard deviation.

[c] s_L = Among-laboratory standard deviation.

[d] s_R = Reproducibility standard deviation.

[e] *P*-value = Homogeneity test of laboratory PODs.

[f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods.

Table 2014.06B. Enrichment protocols using Demi-Fraser broth base without FAC

Sample matrix	Sample size	Primary enrichment, Demi-Fraser broth (no FAC)		
		Enrichment broth volume, mL	Enrichment temperature (±1°C)	Enrichment time, h
Food				
Full-fat cottage cheese	25 g	225	37	24-28
Beef hot dogs	25 g	225	37	24-28
Deli turkey	25 g	225	37	24-28
Cold smoked salmon	25 g	225	37	24-28
Environmental surfaces				
Stainless steel	1 Swab	10	37	26-30
Sealed concrete	1 Sponge	100	37	26-30
Stainless steel, sealed concrete	1 Sponge	225	37	26-30

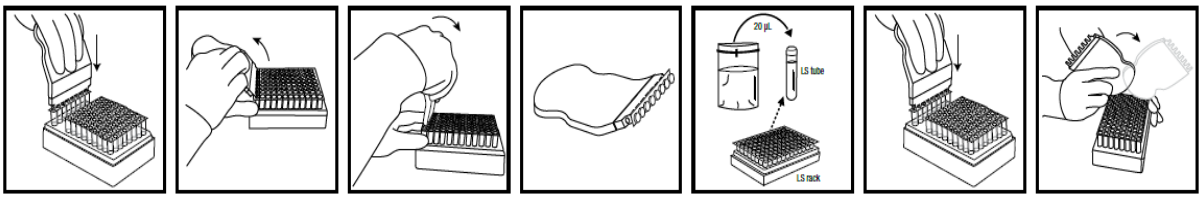


Figure 2014.06A. Transer of enriched sample to lysis solution tube.

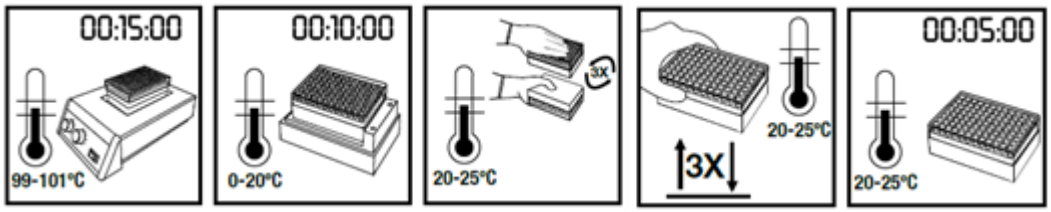


Figure 2014.06B. Sample lysis.

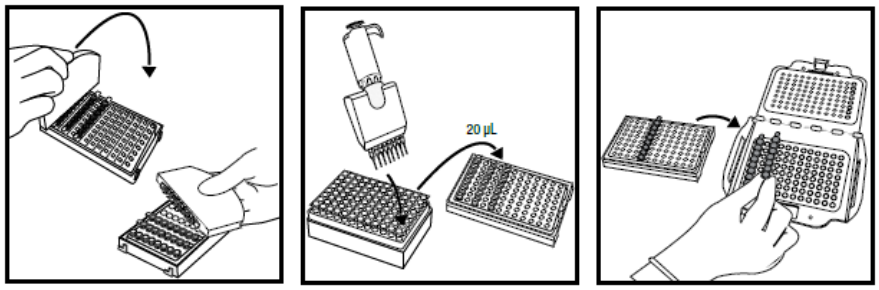


Figure 2014.06C. Transfer of lysate to reagent tube.

Evaluation of 3M™ Molecular Detection Assay (MDA) *Listeria* for the Detection of *Listeria* speciesin SelectedFoods and Environmental Surfaces: Collaborative Study

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The 3M™ Molecular Detection Assay (MDA) *Listeria* is used with the 3M™ Molecular Detection System for the detection of *Listeria* species in food, food-related, and environmental samples after enrichment. The assay utilizes loop-mediated isothermal amplification to rapidly amplify *Listeria*target DNA with high specificity and sensitivity, combined with bioluminescence to detect the amplification. The 3M MDA *Listeria*method was evaluated using an unpaired study design in a multi-laboratory collaborative study and compared to the AOAC® Official Method of Analysis (OMA) 993.12 *Listeria monocytogenes in Milk and Dairy Products* reference method for the detection of *Listeria* species infull fat (4% milk fat) cottage cheese (25 g test portions). A total of 15 laboratories located in the continental United States and Canada participated. Each matrix had 3 inoculation levels: an un-inoculated control level (0 colony forming units (CFU)/test portion), and two levels artificially contaminated with *Listeria monocytogenes,* a low inoculum level (0.2-2 CFU/test portion) and a high inoculum level (2-5 CFU/test portion) using non-heat stressed cells.In total, 792unpaired replicate samples were analyzed. Statistical analysis was conducted according to the Probability of Detection (POD).Results obtained for the low inoculum level test portions produced a dLPOD value with 95% confidence intervals of -0.07, (-0.19, 0.06).Nostatistically significant differences were observed in the number of positive samples detected by the 3M MDA *Listeria*method versustheAOAC OMA method.

Listeria is a Gram-positive, rod shaped bacteriumfound widespread in the environment and one species, *Listeria monocytogenes*, is known to be the causative agent of listeriosis in humans [1]. Due to its high mortality rate, specificallyin susceptible individuals such as older adults, pregnant women, newborns and adults with weakened immune systems, Listeriosis presents itself as an important health problem in the United States, Canada and throughout the world [2].*Listeria's* ability to survive in extreme conditions, such as low temperature and a broadpH range (4.4 to

9.4), can cause severe problems for food manufacturers as the organism can survive cleaning conditions and contaminate food commodities [1, 3]. While less frequent than other food borne pathogens, outbreaks from *Listeria monocytogenes* have been linked to a wide variety of food types, such as raw milks and cheeses, pasteurized dairy products, smoked seafood, ready-to-eat deli meats, hot dogs, and most recently cantaloupes [2]. The presence of other *Listeria* species, such as *Listeria innocua, Listeria welshimeri or Listeria ivanovii*,is often used as an indicator for the possible contamination of *Listeria monocytogenes*[4].The 3M™ Molecular Detection Assay (MDA) *Listeria*method uses loop-mediated isothermal amplification of target nucleic acid sequences to detect *Listeria* in enriched food, feed and environmental samples.The isothermal amplification is a polymerase chain reaction conducted at a constant temperature, eliminating the need for temperature cycling and decreasing the time to results.

The 3M MDA *Listeria* method allows for the rapid and specific detection of *Listeria*species after as little as 24 hoursof enrichment using pre-warmed (37 ± 1°C) Demi Fraser(DF) brothbase (without ferric ammonium citrate (FAC)) or 3M™ Modified *Listeria* Recovery Broth (mLRB). After enrichment, samples are evaluated using the 3M MDA *Listeria* on the 3M™ Molecular Detection System (MDS). Presumptive positive results are reported in real-time while negative results are displayed after completion of the assay (75 minutes).

Prior to the collaborative study, the 3M MDA *Listeria* method was validated according to AOAC Guidelines[5] in a harmonized AOAC® Performance Tested Method℠(PTM) study. The objectiveof the PTM study was to demonstrate that the 3M MDA *Listeria* method could detect *Listeria*on selected environmental surfaces as claimed by the manufacturer.For the 3M MDA *Listeria*PTM evaluation, three(3) matrices were evaluated: stainless steel (sponge in 225 mL 3M mLRB), sealed concrete (sponge in 225 mL 3M mLRB) and plastic (swab in 10 mL 3M mLRB). All other PTM parameters (inclusivity, exclusivity, ruggedness, stability and lot to lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method was awarded PTM certification number 081203 on March 30, 2012.

A method modification and matrix extension study was performedin 2014 with the following matrices: beef hot dogs (25 g), deli turkey (25 g), cold smoked salmon (25 g), full fat cottage cheese (25 g), bagged raw spinach (25 g), whole cantaloupe (whole melon), sealed concrete(sponge in 100 mL and sponge in 225 mL enrichment volume) and stainless steel (swab in 10 mL and sponge in 225 mL enrichment volume) using DF broth base without FAC as the primary enrichment and, where applicable, a secondary enrichment in Fraser broth base without FAC. All other PTM parameters (inclusivity, exclusivity, ruggedness, stability and lot to lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method modification and matrix extension was awarded PTM approval and license number 081203 on June 30, 2014.

The purpose of this collaborative studywas to compare the reproducibility among different laboratories of the 3M MDA *Listeria*method to the AOAC® Official Methodof Analysis 993.12 *Listeria monocytogenes in Milk and Dairy Products*[6] for full fat (4% milk fat) cottage cheese.

Collaborative Study

Study Design

In this collaborative study, one matrix, full fat cottage cheese, was analyzed using 25g test portions. The full fat cottage cheese was obtained from a local retailer and screened for the absence of *Listeria*by the AOAC 993.12 reference method prior to analysis. The matrix was artificially contaminated with non-heat stressed cells of*Listeriamonocytogenes* American Type

Culture Collection (ATCC) 19114at two inoculation levels: a high inoculation level of approximately 2-5 colony-forming units (CFU)/test portion and a low inoculation level of approximately 0.2-2 CFU/test portion. A set of un-inoculated control test portions were also included at 0 CFU/test portion. Twelve replicate samples from each of the three inoculation levels were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by 3M MDA *Listeria* and the AOAC OMA 993.12 reference method due to the different sample enrichment procedures for each method. Additionally, collaborators were sent a 30 g test portion and instructed to conduct atotal aerobic plate count (APC) using 3M™ Petrifilm Aerobic Count Plate (AOAC Official Method 990.12) [7] on the day samples were received for the purpose of determining the total aerobic microbial load.

A detailed collaborative study packet outlining all necessary information related to the study including media preparation, test portion preparation and documentation of results was sent to each collaborating laboratory prior to the initiation of the study. A conference call was conducted to discuss the collaborative study packet and answer any questions from the participating laboratories.

Preparation of Inocula and Test Portions

The *Listeriamonocytogenes* culture used in this evaluation was propagated in 10 mL of Brain Heart Infusion (BHI) broth from a frozen stock culture stored at -70°C at Q Laboratories, Inc. The broth was incubated for 18 ± 0.5 hours at 35 ±1°C. Appropriate dilutions of the culture were prepared based on previously established growth curves for both the low and high inoculation levels. The full fat cottage cheese was inoculated at a low and high inoculation level with the diluted inoculum and mixed thoroughly by hand mixing to ensure an even distribution of microorganisms. The inoculated test product was divided into separate 30 g portions which were packaged into sterile whirl-pak® bags.

To determine the level of *Listeria monocytogenes* in the full fat cottage cheese, a 5-tube most probable number (MPN) was conducted on the day of initiation of analysis. From both the high and low inoculated batches, 5 x50 g test portions, the reference method test portions from the collaborating labs, and 5 x10 g test portions were analyzed. Each test portion was enriched at a 1:10 dilution and evaluated following the AOAC 993.12 reference method. The MPN and 95% confidence intervals were calculated from the high, medium, and low levels using the LCF MPN Calculator, Version 1.6, provided by AOAC RI (www.lcfltd.com/customer/LCFMPNCalculator.exe) [8]. Confirmation of the samples was conducted according to the AOAC OMA 993.12 reference method.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3 digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transportations Association(IATA). Upon receipt, samples were held by the collaborating laboratory at refrigeration temperature (3-5 °C) until the following Monday when analysis was initiated a total of 96 hours after inoculation. All samples were packed with cold packs to target a temperature of < 7°C during shipment. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as 'temperature control'. Participants were instructed to obtain the temperature of this portion upon receipt of the package, document results on the Sample Receipt Confirmation form provided and

fax to the study director.The shipment and hold times (through 120 hours) of the inoculated test material had been verified as a quality control measure prior to study initiation.

Test Portion Analysis

Each collaborator received 72 test portions of full fat cottage cheese (12 high inoculum, 12 low inoculum and 12 uninoculated controls for each method). Collaborators followed the appropriate preparation and analysis protocol according to the method specified for the matrix.
For the analysis of the test portions by the 3M MDA *Listeria* method, a 25 g portion was enriched with 225 mL of pre-warmed (37 ±1°C) DF broth base without FAC, homogenized for 2 ± 0.5 minutes and incubated for 26± 2 hours at 37 ±1°C. Following enrichment, samples were assayed by the 3M MDA *Listeria* method and confirmed following the standard reference method by streaking an aliquot of the primary enrichment onto Oxford Agar (OXA). Presumptive positive samples were streaked for isolation on Trypticase Soy Agar with yeast extract (TSA/ye), verified morphologically by Gram stain and biochemically confirmed by hemolysis testing and by VITEK 2 GP Biochemical Identification method (AOAC OMA 2012.02) [9]or API Listeria Identification System biochemical test kits. Laboratories utilizing API Listeria kits were also required to conduct catalase and oxidase tests.

For samples analyzed using the AOAC OMA 993.12 reference method, 25 g test portions were enriched in pre-warmed (45± 2°C) selective enrichment broth, homogenized for 2± 0.5 minutes and incubated at 30 ± 2°C for 48± 2 hours. Samples were streaked onto OXA and presumptive positive samples were streaked for isolation onto TSA/ye. Colonies from TSA/ye were verified morphologically by Gram stain and biochemically confirmed by hemolysis test and by VITEK 2 GP Biochemical Identification method or API Listeria biochemical test kits. Laboratories utilizing API Listeria kits were also required to conductcatalaseand oxidase tests.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and the 3M MDA *Listeria* method on the data sheets provided. The data sheets were submitted to the study director at the end of testing for analysis. The results of each test portion for each sample were compiled by the study director and the 3M MDA *Listeria* results were compared to the reference method for statistical analysis. Data for each test portion size was analyzed using the probability of detection (POD) [10]. The probability of detection (POD) was calculated as the number of positive outcomes divided by the total number of trials. The POD was calculated for the candidate presumptive results, $POD_{CP,}$ the candidate confirmatory results (including false negative results), POD_{CC}, the difference in the candidate presumptive and confirmatory results, $dPOD_{CP,}$ presumptive candidate results that confirmed positive (excluding false negative results), $POD_{C,}$ the reference method, POD_R, and the difference in the confirmed candidate and reference methods, $dPOD_C$. A dLPOD confidence interval not containing the point zero would indicate a statistically significant difference between the 3M MDA *Listeria*and the AOAC OMA 993.12 reference methods at the 5 % probability level.. In addition to POD, the repeatability standard deviation (s_r), the among laboratory repeatability standard deviation (s_L), the reproducibility standard deviation ($s_{R)}$ and the P_T value were calculated. The s_r provides the variance of data within one laboratory, the s_L provides the difference in standard deviation between laboratories

and the s_R provides the variance in data between different laboratories. The P_T value provides information on the homogeneity test of laboratory PODs [11].

AOAC Official Method 2014.xxx
***Listeria* speciesin Selected Foods and Environmental Surfaces**
3M™ Molecular Detection Assay (MDA) *Listeria* Method
First Action 2014

(Applicable to detection of *Listeria* species inselected foods including beef hot dogs (25 g), deli turkey (25 g), cold smoked salmon (25 g), full fat cottage cheese (25 g), and two environmental surfacessealed concrete (sponge in 100 mL and sponge in 225 mL enrichment volume) and stainless steel (swab in 10 mL and sponge in 225 mL enrichment volume) and plastic (swab in 10 mL) enriched in Demi-Fraser broth base
*See Table **2014.1** for a summary of results of the inter-laboratory study.*
*See Tables **2014.2** of the Supplementary Materialsfor detailed of results of the inter-laboratory study*

A. Principle

3M™ Molecular Detection Assay (MDA) *Listeria*method is intended for use with the 3M™ Molecular Detection System for the rapid and specific detection of *Listeria*spp. in selected foodsand environmental surfaces. The 3M MDA *Listeria* test uses isothermal amplification of unique DNA target sequences with high specificity, efficiency, rapidity and bioluminescence to detect the amplified sequences. Presumptive positive results are reported in real-time while negative results are displayed after the assay is completed. Samples areenrichedin pre-warmed 3M™Demi Fraser (DF) broth base, which does not contain Ferric Ammonium Citrate (FAC).

B. Apparatus and Reagents

Items (b)-(g) are available as the 3M™ Molecular Detection Assay (MDA) *Listeria* kit from 3M Food Safety (St. Paul, MN 55144-1000, USA).

- **(a)** *3M Molecular Detection System* – Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
- **(b)** *3M Molecular Detection Assay Listeria reagent tubes*- 12 strips of 8 tubes. Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
- **(c)** *Lysis Solution (LS) tubes* – 12 strips of 8 tubes.

(d) *Extra caps* – 12 strips of 8 caps
(e) *Negative control (NC)* - One vial (2 mL).
(f) *Reagent Control* – 8 reagent tubes
(g) *Quick Start Guide*
(h) *3M™ Molecular Detection Speed Loader Tray* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
(i) *3M™ Molecular Detection Chill Block Tray and Chill block insert* - Available from 3M™ Food Safety (St. Paul, MN 55144-1000, USA).
(j) *3M™ Molecular Detection Heat Block Insert* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
(k) *3M™ Molecular Detection Cap/Decap Tool for Reagent tubes* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
(l) *3M™ Molecular Detection Cap/Decap Tool for Lysis tubes* – Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
(m) *Empty lysis tube rack* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
(n) *Empty reagent tube rack* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
(o) *Demi-Fraser Broth Base*– Available from 3M Food Safety (St. Paul, MN 55144-1000, USA). (Formulation equivalent to ISO 11290-1:1996)
(p) *Disposable pipette* – capable of 20 µL
(q) *Multi*-channel (8-channel) pipette - capable of 20 µL
(r) *Sterile filter tip pipette tips* - capable of 20 µL
(s) *Filter Stomacher® bags* – Seward or equivalent.
(t) *Stomacher®*– Seward or equivalent.
(u) *Thermometer* – calibrated range to include 100 ± 1°C
(v) *Dry double block heater unit or water bath* – capable of maintaining 100 ± 1°C
(w) *Incubators.* – Capable of maintaining 37 ± 1°C
(x) *Freezer* – capable of maintaining -10 to -20 °C, for storing the 3M Molecular Detection Chill Block Tray
(y) *Refrigerator* – capable of maintaining 2-8°C, for storing the 3M Molecular Detection Assay
(z) *Computer* – compatible with the 3M™ Molecular Detection Instrument

C. General Instructions

(a) Store the 3M Molecular Detection Assay *Listeria* at 2-8°C. Do not freeze. Keep kit away from light during storage. After opening the kit, check that the foil pouch is undamaged. If the pouch is damaged, do not use. After opening, unused reagent tubes should always be stored in the re-sealable pouch with the desiccant inside to maintain stability of the lyophilized reagents. Store resealed pouches at 2-8°C for no longer than 1 month. Do not use 3M Molecular Detection Assay *Listeria* past the expiration date.

(b) The 3M Molecular Detection Instrument is intended for use with samples that have undergone heat treatment during the assay lysis step, which is designed to destroy organisms present in the sample. Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should NOT be inserted into the 3M Molecular Detection Instrument.

(c) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

Safety Precautions

Periodically decontaminate laboratory benches and equipment (pipettes, cap/decap tools, etc…) with a 1- 5% (v:v in water) household bleach solution or DNA removal solution.

Listeria monocytogenes is of particular concern for pregnant women, the aged and the infirmed. It is recommended that these concerned groups avoid handling this organism.After use, the enrichment medium and the 3M Molecular Detection Assay *Listeria*tubes can potentially contain pathogenic materials. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal.

Ethanol used in the method is flammable and caution should be used. Consult MSDS for additional information.

D. Sample Enrichment

(a) Pre-warmDF broth base without FAC,to 37 ±1°C.
(b) Aseptically combine the enrichment medium and sample following the procedures in Table Abelow. For all meat and highly particulate samples, the use of filter bags is recommended. Homogenize thoroughly (Stomacher, blender) for 2 ± 0.5 minutes Incubate at 37 ±1°C.

Table A: Enrichment protocols using **Demi-Fraser Broth Base without FAC**

	Sample Matrix	Sample Size	Primary Enrichment Demi-Fraser Broth (no FAC)					
			Enrichment Broth Volume (mL)	Enrichment Temperature (±1°C)	Enrichment Time (hr)			

	Full fat cottage cheese		25 g	225	37	24-28			
	Beef hot dogs		25 g	225	37	24-28			
	Deli turkey		25 g	225	37	24-28			
	Cold smoked salmon		25 g	225	37	24-28			
	Environmental Surfaces	Stainless steel	1 swab	10	37	26-30			
		Sealed Concrete	1 sponge	100					
		Stainless steel, sealed concrete	1 sponge	225					

E. Preparation of the 3M™ Molecular Detection Speed Loader Tray

(a) Wet a cloth or paper towel with a 1-5% (v:v in water) household bleach solution and wipe the 3M™ Molecular Detection Speed Loader Tray.
(b) Rinse the 3M Molecular Detection Speed Loader Tray with water.
(c) Use a disposable towel to wipe the 3M Molecular Detection Speed Loader Tray dry.
(d) Ensure the 3M Molecular Detection Speed Loader Tray is dry before use

F. Preparation of the 3M™ Molecular DetectionChill Block Insert

Before using the 3M™ Molecular Detection Chill Block Insert, ensure it has been stored on the 3M™ Molecular Detection Chill Block Tray in the freezer (-10 to -20°C) for a minimum of 2 hours before use. When removing the 3M Molecular Detection Chill Block Insert from the freezer for use, remove it and the 3M Molecular Detection Chill Block Tray together. Use the 3M Molecular Detection Chill Block Insert /3M Molecular Detection Chill Block Tray within 20 minutes.

G. Preparation of the 3M™ Molecular Detection Heat Block Insert

Place the 3M™ Molecular Detection Heat Block Insert in a dry double block heater unit. Turn on the dry block heater unit and set the temperature to allow the 3M Molecular Detection Heat Block Insert to reach and maintain a temperature of 100 ±1°C.

NOTE: Depending on the heater unit, allow approximately 30-50 minutes for the 3M Molecular Detection Heat Block Insert to reach temperature. Using a calibrated thermometer, verify that the 3M Molecular Detection Heat Block Insert is at 100 ±1°C.

H. PREPARATION OF THE 3M MOLECULAR DETECTION INSTRUMENT

1. Launch the 3M™ Molecular Detection Software and log in.
2. Turn on the 3M Molecular Detection Instrument.
3. Create or edit a run with data for each sample. Refer to the 3M Molecular Detection System User Manual for details.

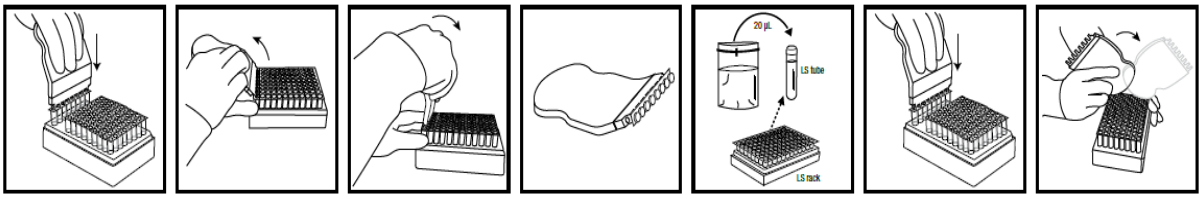
NOTE: The 3M Molecular Detection Instrument must reach and maintain temperature of 60°C before inserting the 3M Molecular Detection Speed Loader Tray with reaction tubes. This heating step takes approximately 20 minutes and is indicated by an ORANGE light on the instrument's status bar. When the instrument is ready to start a run, the status bar will turn GREEN.

I. LYSIS

1. Allow the lysis solution(LS) tubes to warm up to room temperature by setting the rack on the laboratory bench for 2 hours [(a)].
2. Remove the enrichment broth from the incubator and gently agitate the contents.
3. One LS tube is required for each sample and the Negative Control (NC) sample.
   - 3.1 LS tube strips can be cut to desired LS tube number. Select the number of individual LS tubes or 8-tube strips needed. Place the LS tubes in an empty rack.
   - 3.2 To avoid cross-contamination, decap one LS tubes strip at a time and use a new pipette tip for each transfer step.
4. Transfer enriched sample to LS tubes as described below:

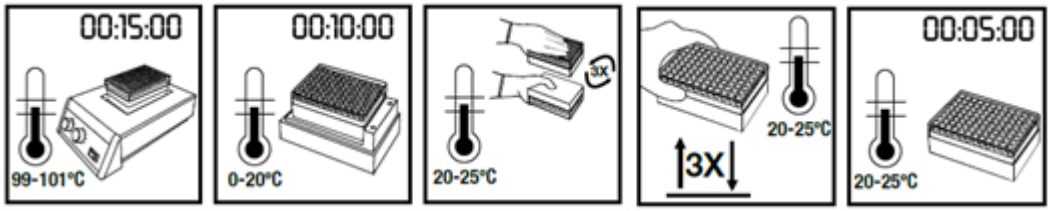
Transfer each enriched sample into individual LS tube **first**. Transfer the NC **last**.

- 4.1 Use the 3M™ Molecular Detection Cap/Decap Tool-Lysis to decap one LS tube strip - one strip at a time. Set the tool with cap attached aside on a clean surface.
- 4.2 Transfer 20 µL of sample into a LS tube.
- 4.3 Repeat step 4.2 until each individual sample has been added to a corresponding LS tube in the strip
- 4.4 Use the 3M Molecular Detection Cap/Decap Tool-Lysis to re-cap the LS tube strip. Use the rounded side of the tool to apply pressure in a back and forth motion ensuring that the cap is tightly applied.



- 4.5 Repeat steps 4.1 to 4.4 as needed, for the number of samples to be tested
- 4.6 When all samples have been transferred, then transfer **<u>20 µL of NC</u> into a LS tube**. Use the 3M Molecular Detection Cap/Decap Tool-Lysis tool to re-cap the LS tube.
- 4.7 Cover the rack of LS tubes with the rack lid and firmly invert 3-5 times to mix. **Suspension has to flow freely inside the tube.**

5. Verify that the temperature of the 3M Molecular Detection Heat Block Insert is at 100 ±1°C. Place the rack of LS tubes in the 3M Molecular Detection Heat Block Insert and heat for 15 ±1 minutes [(b)]. Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should NOT be inserted into the 3M Molecular Detection Instrument.
6. Remove the rack of LS tubes from the heating block and allow to cool in the 3M Molecular Detection Chill Block Insert for 10 ±1 minutes [(c)]. **Remove the rack lid during incubation on the 3M Molecular Detection Chill Block Insert**.
7. Remove the rack of LS tubes from the 3M Molecular Detection Chill Block Insert/ 3M Molecular Detection Chill Block Tray system. Replace the lid on the rack of LS tubes and firmly invert 3-5 times to mix. **Suspension has to flow freely inside the tube.**
8. Firmly tap the lysis tubes rack on the laboratory bench 3-5 times.
9. Place the rack on the laboratory bench. Let it sit undisturbed for at least 5 minutes to allow the resin to settle. **Do not mix or disturb the resin at the bottom of the tube**.



(a) Alternatives to equilibrate the LS tubes to room temperature are to incubate the LS tubes in a 37 ±1°C incubator for 1 hour or at room temperature overnight (16-18 hours).

(b) An alternative to using dry heat for the lysis step is to use a water bath at 100 ±1°C. Ensure that sufficient water is used to cover up to the liquid level in the LS tubes. Place the rack of LS tubes in the water bath at 100 ±1°C and heat for 15 ±1 minutes.

(c) The LS solution may freeze when processing less than 48 LS tubes. Freezing of the LS solution will not affect your test. If freezing is observed, allow the LS tubes to thaw for 5 minutes before mixing.

J. AMPLIFICATION

1. One Reagent tube is required for each sample and the NC.
   - 1.1 Reagent tubes strips can be cut to desired tube number. Select the number of individual Reagent tubes or 8-tube strips needed.
   - 1.2 Place Reagent tubes in an empty rack.
   - 1.3 Avoid disturbing the reagent pellets from the bottom of the tubes.
2. Select 1 Reagent Control (RC) tube and place in rack.
3. To avoid cross-contamination, decap one Reagent tubes strip at a time and use a new pipette tip for each transfer step.
4. Transfer lysate to Reagent tubes and RC tube as described below:

> Transfer each sample lysate into individual Reagent tubes **first** followed by the NC. Hydrate the RC tube **last**.
> **WARNING:** Care must be taken when pipetting LS, as carry-over of the resin may interfere with amplification.

   - 4.1 Use the 3M™ Molecular Detection Cap/Decap Tool-Reagent to decap the Reagent tubes –one Reagent tubes strip at a time. Discard cap.
   - 4.2 Transfer 20 µL of Sample lysate from the upper portion of the fluid in the LS tube into corresponding Reagent tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down 5 times.

4.3 Repeat step 4.2 until individual Sample lysate has been added to a corresponding Reagent tube in the strip.

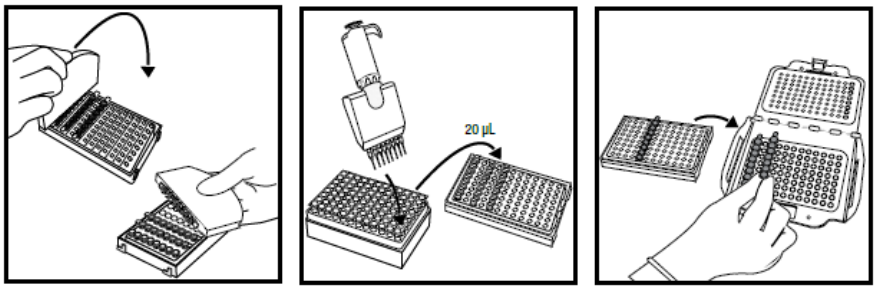
4.4 Cover the Reagent tubes with the provided extra cap and use the rounded side of the 3M Molecular Detection Cap/Decap Tool-Reagent to apply pressure in a back and forth motion ensuring that the cap is tightly applied.

4.5 Repeat steps 4.1 to 4.4 as needed, for the number of samples to be tested.

4.6 When all sample lysates have been transferred, repeat 4.1 to 4.4 to transfer 20 µL of NC lysate into a Reagent tube.

4.7 Transfer **20 µL of NC lysate into a RC tube**. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down 5 times.

5. Load capped tubes into a clean and decontaminated 3M Molecular Detection Speed Loader Tray. Close and latch the 3M Molecular Detection Speed Loader Tray lid.



6. Review and confirm the configured run in the 3M Molecular Detection Software.
7. Click the Start button in the software and select instrument for use. The selected instrument's lid automatically opens.
8. Place the 3M Molecular Detection Speed Loader Tray into the 3M Molecular Detection Instrument and close the lid to start the assay. Results are provided within 75 minutes, although positives may be detected sooner.
9. After the assay is complete, remove the 3M Molecular Detection Speed Loader Tray from the 3M Molecular Detection Instrument and dispose of the tubes by soaking in a 1-5% (v:v in water) household bleach solution for 1 hour and away from the assay preparation area.

NOTICE: To minimize the risk of false positives due to cross-contamination, never open reagent tubes containing amplified DNA. This includes Reagent Control, Reagent and Matrix Control tubes. Always dispose of sealed reagent tubes by soaking in a 1-5% (v:v in water) household bleach solution for 1 hour and away from the assay preparation area.

K. RESULTS AND INTERPRETATION

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A Positive or Negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real-time while Negative and Inspect results will be displayed after the run is completed. Presumptive positive results should be confirmed using your preferred method or as specified by the FDA/BAM, the USDA/FSIS-MLG, the AOAC OMA 993.12 or the ISO 11290 methods starting from the 3M primary enrichment, followed by transfer to a secondary enrichment or direct plating onto media through confirmation of isolates using appropriate biochemical and serological methods.

NOTE: Even a negative sample will not give a zero reading as the system and 3M Molecular Assay *Listeria*amplification reagents have a "background" relative light unit (RLU).

In the rare event of any unusual light output, the algorithm labels this as “Inspect.” 3M recommends the user to repeat the assay for any Inspect samples. If the result continues to be Inspect, proceed to confirmation test using your preferred method or as specified by local regulations.

Results of Collaborative Study

For this collaborative study, the 3M Molecular Detection Assay (MDA) *Listeria* method was comparedto the AOAC 993.12 reference method for full fat cottage cheese. A total of 15 laboratories throughout the United States and Canada participated in this study, with 13 laboratories submitting data for the full fat cottage cheese. Each laboratory analyzed 36 test portions for each method: 12 inoculated with a high level of *Listeria*, 12 inoculated with a low level of *Listeria*, and 12 un-inoculated controls. The 3M™ MDA *Listeria* method produced 199 presumptive positive results with 196 confirming positive by traditional confirmation. There were 205 confirmed positives by the reference method.
A background screen of the matrix indicated an absence of indigenous *Listeria*species. For each matrix, the level of *Listeria* was determined by MPN determination on the day of initiation of analysis by the coordinating laboratory. The individual laboratory and sample results are presented in Table 2. Table 2014.1 summarizes the inter-laboratory results for all foods tested, including POD statistical analysis [11]. As per criteria outlined in Appendix J of the AOAC Validation Guidelines, fractional positive results were obtained.Detailed results for each laboratory are presented in Tables 2014.2 and Figures 1A-1Bof the Supplementary Materials. The results for each collaborating laboratory’s 3M Petrifilm Aerobic Count Plate(OMA 990.12) for full fat cottage cheeseare presented in Table 2014.3 of the Supplementary Materials.

Full Fat Cottage Cheese (25 g Test Portions)

Full fat cottage cheesetest portions were inoculated at a low and high level and were analyzed (Table 2) for the detection of *Listeria* spp. Un-inoculated controls were included in each analysis. Laboratories 4 and 5 did not submit results to the coordinating laboratory. Laboratories 6 and 13reported deviations in the protocol (laboratory 6 incorrectly incubated their MDA test portions at 30°C instead for 48 hours instead of the required 37°C for 24 hours; laboratory 13 confirmed all colony growth regardless of supplementary tests, (Gram stain, catalase reaction) indicated that the organism would not be classified as *Listeria* (Gram negative or Gram positive with spores, catalase negative))and results from these laboratories were excluded from the statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.80 CFU/test portion (0.63,1.00) for the low level and 4.83 CFU/test portion (3.30, 7.70) for the high level.
For the high level, 132 out of 132 test portions (POD_{CP} of 1.00) were reported as presumptive positive by the 3M MDA *Listeria*method with all 132 test portions (POD_{CC} of 1.00) confirming positive.Based on the valid data submitted from each of the collaborating laboratories, 0 false negative results or false positive results were obtained resulting in 132 confirmed positives (POD_C of 1.00). For the low level, 67 out of 132 test portions (POD_{CP} of 0.51) were reported as presumptive positive by the 3M MDA *Listeria*method with 64 test portions (POC_{CC} of 0.48) confirming positive.Based on the valid data submitted from each of the collaborating laboratories, 3 false positive results were obtained resulting in 64 confirmed positives (POD_C of 0.48). For the un-inoculated controls, 1 out of 132 samples (POD_{CP} of 0.01) produced a presumptive positive result by the 3M MDA *Listeria*method with all 132 test portions (POD_{CC} of 0.00) confirming negative.. Based on the valid data submitted from each of the collaborating laboratories, 0 false negative results and 0 false positive results were obtained resulting in 0

confirmed positives (POD_C of 0.00). For test portions analyzed by the AOAC 993.12 Method, 132 out of 132 high inoculum test portions and 73 out of 132 low inoculum test portions confirmed positive. For the un-inoculated controls, 0 out of 132 test portions confirmed positive. For the low level inoculum, a $dLPOD_C$ value of -0.07 with 95% confidence intervals of (-0.19, 0.06) were obtained between the 3M MDA *Listeria*method and the AOAC OMA 993.12 method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.01 with 95% confidence intervals of (-0.12, 0.13) were obtained between presumptive and confirmed 3M MDA *Listeria* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high level inoculum, a $dLPOD_C$ value of 0.00 with 95% confidence intervals of (-0.03, 0.03) were obtained between the 3M MDA *Listeria*method and the AOAC OMA 993.12 method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (-0.03, 0.03) were obtained between presumptive and confirmed 3M MDA *Listeria* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table 2014.2 and Figures 1A-1B of the Appendix.

Discussion

No negative feedback was provided by the collaborating laboratories in regards to the performance of the 3M MDA *Listeria*. Several laboratories reported difficulty in isolating and identifying*Listeria* colonies on Oxford agar(OXA)from samples enriched in the DF brothbase (without FAC) when compared to samples enriched in the AOAC OMA 991.12 selective enrichment broth. This may be related to differences in formulation between the two enrichments. The AOAC OMA 993.12 enrichment brothis designed to reduce the background flora on OXA and is more selective than DF broth base (without FAC). In some instances, this level of selectivity may cause stress on*Listeria* cells thus requiring a longer enrichment time to reach a detectable level.

Based on the data submitted, 2 laboratories, laboratory 6 and laboratory 13, were removed from statistical consideration for the full fat cottage cheeseDuring analysis,laboratory 6 did not follow the approved incubation time and temperature for the candidate method (samples were incubated for 48 hours at 30°C and the validated enrichment time and temperature are 24 to 28 hours at 37°C.) andlaboratory 13 confirmed growth from all plates, regardless of supplementary tests that would have precluded confirmation via API *Listeria.* Due to this fact, all samples confirmed via API *Listeria* produced a *Listeria* species result even if Gram stain reaction (Gram negative), motility reaction (negative), catalase reaction (negative) and oxidase reaction (positive) would indicate the organism is not of the genus *Listeria*.

During the analysisof the full fat cottage cheese,3false positive results were obtainedout of 396 test portions analyzed with the candidate method. The 3M MDA *Listeria* correctly identified whether a test portion was positive or negative more than 99.2% of the time (false positive rate of 0.8%). For full fat cottage cheese, the collaborative study indicated thatno statistically significant difference between the candidate method and the reference method or the presumptive and confirmed results of the candidate method was obtainedwhen using the POD statistical model.

Recommendations

It is recommended that the 3M Molecular Detection Assay *Listeria*method be adopted as Official First Action status for the detection of *Listeria*species in selected foods including beef hot dogs (25 g), deli turkey (25 g), cold smoked salmon (25 g), full fat cottage cheese (25 g), and two environmental surfaces sealed concrete (sponge in 100 mL and sponge in 225 mL enrichment volume) and stainless steel (swab in 10 mL and sponge in 225 mL enrichment volume) and plastic (swab in 10 mL) enriched in Demi-Fraser broth base.

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(11) Least Cost Formulations, Ltd., AOAC Binary Data Interlaboratory Study Workbook (2011), http://lcfltd.com/aoac/aoac-binary-v2-3.xls (Accessed September, 2014)

Table 1: Participation of each Collaborating Laboratory[a]

Lab	Full Fat Cottage Cheese[a]
1	Y
2	Y
3	Y
4	Y[c]
5	Y[c]
6	Y[b]
7	Y
8	Y
9	Y
10	Y
11	Y
12	Y
13	Y[b]
14	Y
15	Y

[a] Y= Collaborator analyzed the food type;
[b] Results were not used in statistical analysis due to laboratory error
[c]Results were not submitted to the coordinating laboratory

Table 2. Individual Collaborator Results for Full Fat Cottage Cheese (4% Milk Fat)

	High-Level Test Portions												Low Level Test Portions												Un-inoculated Test Portions											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
3M MDA *Listeria*																																				
Lab																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	-[c]	+	+	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+	+	+	-[c]	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
4[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
6[b]	+	+	+	+	+	+	+	+	+	+	+	+	+[d]	+[d]	+[d]	+[d]	+	+	+	+	+	+[d]	+	+	+[d]	+[d]	+[d]	+[d]	+	+[d]	+[d]	+[d]	+[d]	+[d]	+[d]	+[d]
7	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-[c]
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13[b]	+[d]	+[d]	+[d]	+[d]	+[d]	+[d]	+[d]	+	+	+[d]	+[d]	+[d]	+[d]	-	+[d]	+	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
15	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
AOAC OMA 993.12																																				
Lab																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
6[b]	+	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
8	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
10	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
12	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
13[b]	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
15	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-

[a] += *Listeria* species were detected in samples; -=*Listeria* species were not detected in sample; n/a- lab did not participate in this matrix or results were not received

[b] Results were not used in statistical analysis due to laboratory error; [c] Sample was presumptive positive on 3M MDA *Listeria* but confirmed negative indicating a false positive result; [d]Sample was presumptive negative on 3M MDA *Listeria* but confirmed positive indicating a false negative result

1

2

3

4

Table 2014.1A:Summary of Results for the Detection of *Listeria* inFull Fat Cottage Cheese (25g)

Method[a]	**3M™ MDA™*Listeria***		
Inoculation Level	**Uninoculated**	**Low**	**High**
Candidate Presumptive Positive/ Total # of Samples Analyzed	1/132	67/132	132/132
Candidate Presumptive POD (CP)	0.01 (0.00, 0.04)	0.51 (0.42, 0.60)	1.00 (0.97, 1.00)
s_r[b]	0.09 (0.08, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.04)	0.00 (0.00, 0.17)	0.00 (0.00, 0.16)
s_R[d]	0.09 (0.08, 0.10)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P Value[e]	0.4338	0.8931	1.0000
Candidate Confirmed Positive/ Total # of Samples Analyzed	0/132	64/132	132/132
Candidate Confirmed POD (CC)	0.00 (0.00, 0.03)	0.48 (0.40, 0.57)	1.00 (0.97, 1.00)
s_r[b]	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.15)	0.00 (0.00, 0.16)
s_R[d]	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P Value[e]	1.0000	0.8762	1.0000
Positive Reference Samples/ Total # of Samples Analyzed	0/132	73/132	132/132
Reference POD	0.00 (0.00, 0.03)	0.55 (0.47, 0.64)	1.00 (0.97, 1.00)
s_r[b]	0.00 (0.00, 0.16)	0.50 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.18)	0.00 (0.00, 0.16)
s_R[d]	0.00 (0.00, 0.23)	0.50 (0.45, 0.52)	0.00 (0.00, 0.23)
P Value[e]	1.0000	0.6678	1.0000
dLPOD (Candidate vs. Reference)[f]	0.00 (-0.03, 0.03)	-0.07 (-0.19, 0.06)	0.00 (-0.03, 0.03)
dLPOD (Candidate Presumptive vs. Candidate Confirmed)[f]	0.01 (-0.02, 0.04)	0.01 (-0.12, 0.13)	0.00 (-0.03, 0.03)

[a] Results include 95% Confidence Intervals, [r] Repeatability Standard Deviation, [c] Among-Laboratory Standard Deviation, [d] Reproducibility Standard Deviation
[e] P Value = Homogeneity test of laboratory PODs; [f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods

Supplementary Materials

Table 2014.2A: Comparative Results for the Detection of *Listeria* in Full Fat Cottage Cheese (4% Milk Fat) by the 3M™ MDA *Listeria* Method vs. AOAC OMA 993.12 in a Collaborative Study (Un-inoculated Control)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD (CP)	N	X	POD (CC)	N	X	POD (C)	N	X	POD (R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		2	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		3	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		4[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[a]	12	1	0.08	12	12	1.00	12	1	0.08	12	0	0.00	0.08	1.00
		7	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		8	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		9	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		10	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		11	12	1	0.08	12	0	0.00	12	0	0.00	12	0	0.00	0.08	0.00
		12	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		13[a]	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		14	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		15	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
Estimate		All	132	1	0.01	132	0	0.00	132	0	0.00	132	0	0.00	0.00	0.01
LCL					0.00			0.00			0.00			0.00	-0.03	-0.02
UCL					0.04			0.03			0.03			0.03	0.03	0.04
s_r[b]					0.09			0.00			0.00			0.00		
LCL					0.08			0.00			0.00			0.00		
UCL					0.16			0.16			0.16			0.16		
s_L[c]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.04			0.16			0.16			0.16		
s_R[d]					0.09			0.00			0.00			0.00		
UCL					0.08			0.00			0.00			0.00		
LCL					0.10			0.23			0.23			0.23		
P_T[e]					0.4338			1.0000			1.0000			1.0000		

[a] Results were not used in statistical analysis due to deviation from testing protocol.

[b] Repeatability Standard Deviation, [c] Among-Laboratory Standard Deviation, [d] Reproducibility Standard Deviation, [e] P_T Value =Homogeneity test of laboratory PODs

LCL – Lower Confidence Limit; UCL – Upper Confidence Limit;NA- laboratory results were not received

Table 2014.2A (cont'd.): Comparative Results for the Detection of *Listeria* in Full Fat Cottage Cheese (4% Milk Fat) by the 3M™ MDA *Listeria* Method vs. AOAC OMA 993.12 in a Collaborative Study (Low Level)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD(CP)	N	X	POD(CC)	N	X	POD(C)	N	X	POD(R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	6	0.50	12	4	0.33	12	4	0.33	12	6	0.50	-0.17	0.17
		2	12	7	0.58	12	7	0.58	12	7	0.58	12	7	0.58	0.00	0.00
		3	12	8	0.67	12	7	0.58	12	7	0.58	12	7	0.58	0.09	0.00
		4[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[a]	12	7	0.58	12	12	1.00	12	7	0.58	12	2	0.17	0.41	-0.42
		7	12	4	0.33	12	4	0.33	12	4	0.33	12	10	0.83	-0.50	0.00
		8	12	7	0.58	12	7	0.58	12	7	0.58	12	7	0.58	0.00	0.00
		9	12	4	0.33	12	4	0.33	12	4	0.33	12	8	0.67	0.34	0.00
		10	12	6	0.50	12	6	0.60	12	6	0.60	12	7	0.58	0.08	0.00
		11	12	6	0.50	12	6	0.60	12	6	0.60	12	5	0.42	0.08	0.00
		12	12	6	0.50	12	6	0.50	12	6	0.50	12	5	0.42	0.08	0.00
		13[a]	12	5	0.42	12	7	0.58	12	5	0.42	12	7	0.58	0.00	-0.16
		14	12	6	0.50	12	6	0.50	12	6	0.50	12	6	0.50	0.00	0.00
		15	12	7	0.58	12	7	0.58	12	7	0.58	12	5	0.42	0.16	0.00
Estimate	0.80	All	132	67	0.51	132	64	0.48	132	64	0.48	132	73	0.55	-0.07	0.01
LCL	0.63				0.42			0.40			0.40			0.47	-0.19	-0.12
UCL	1.00				0.60			0.57			0.57			0.64	0.06	0.13
s_r[b]					0.51			0.51			0.51			0.50		
LCL					0.45			0.45			0.45			0.45		
UCL					0.52			0.52			0.52			0.52		
s_L[c]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.17			0.15			0.15			0.18		
s_R[d]					0.51			0.51			0.51			0.50		
UCL					0.46			0.46			0.46			0.45		
LCL					0.52			0.52			0.52			0.52		
P_T[e]					0.8931			0.8762			0.8762			0.6678		

[a] Results were not used in statistical analysis due to deviation from testing protocol

[b] Repeatability Standard Deviation, [c] Among-Laboratory Standard Deviation, [d] Reproducibility Standard Deviation, [e] P_T Value =Homogeneity test of laboratory PODs

LCL – Lower Confidence Limit; UCL – Upper Confidence Limit; NA- laboratory results were not received

Table 2014.2A (cont'd): Comparative Results for the Detection of *Listeria*in Full Fat Cottage Cheese (4% Milk Fat) by the 3M™ MDA *Listeria* Method vs. AOAC OMA 993.12 in a Collaborative Study (High Level)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD(CP)	N	X	POD(CC)	N	X	POD(C)	N	X	POD(R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		2	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		3	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		4[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[a]	12	12	1.00	12	12	1.00	12	12	1.00	12	7	0.58	0.42	0.00
		7	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		8	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		9	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		10	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		11	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		12	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		13[a]	12	2	0.17	12	2	0.17	12	12	1.00	12	12	1.00	-0.83	0.00
		14	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		15	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
Estimate	4.83	All	132	132	1.00	132	132	1.00	132	132	1.00	132	132	1.00	0.00	0.00
LCL	3.30				0.97			0.97			0.97			0.97	-0.03	-0.03
UCL	7.07				1.00			1.00			1.00			1.00	0.03	0.03
s_r[b]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.16			0.16			0.16			0.16		
s_L[c]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.16			0.16			0.16			0.16		
s_R[d]					0.00			0.00			0.00			0.00		
UCL					0.00			0.00			0.00			0.00		
LCL					0.23			0.23			0.23			0.23		
P_T[e]					1.0000			1.0000			1.0000			1.0000		

[a] Results were not used in statistical analysis due to deviation from testing protocol.

[c] Repeatability Standard Deviation, [d] Among-Laboratory Standard Deviation, [e] Reproducibility Standard Deviation, [f] P_T Value =Homogeneity test of laboratory PODs

LCL – Lower Confidence Limit; UCL – Upper Confidence Limit; NA- laboratory results were not received

Table 2014.3: Results of Aerobic Plate Count for Collaborating Laboratories

Lab	Full Fat Cottage Cheese (CFU/g)
1	1.0×10^1
2	7.2×10^3
3	1.0×10^1
4	Not Reported
5	Not Reported
6	1.5×10^1
7	<10
8	3.0×10^2
9	1.2×10^2
10	4.2×10^3
11	<10
12	5.0×10^1
13	8.5×10^1
14	3.2×10^3
15	1.2×10^2

Figure 1A: POD Values of Candidate Method vs. Reference Method

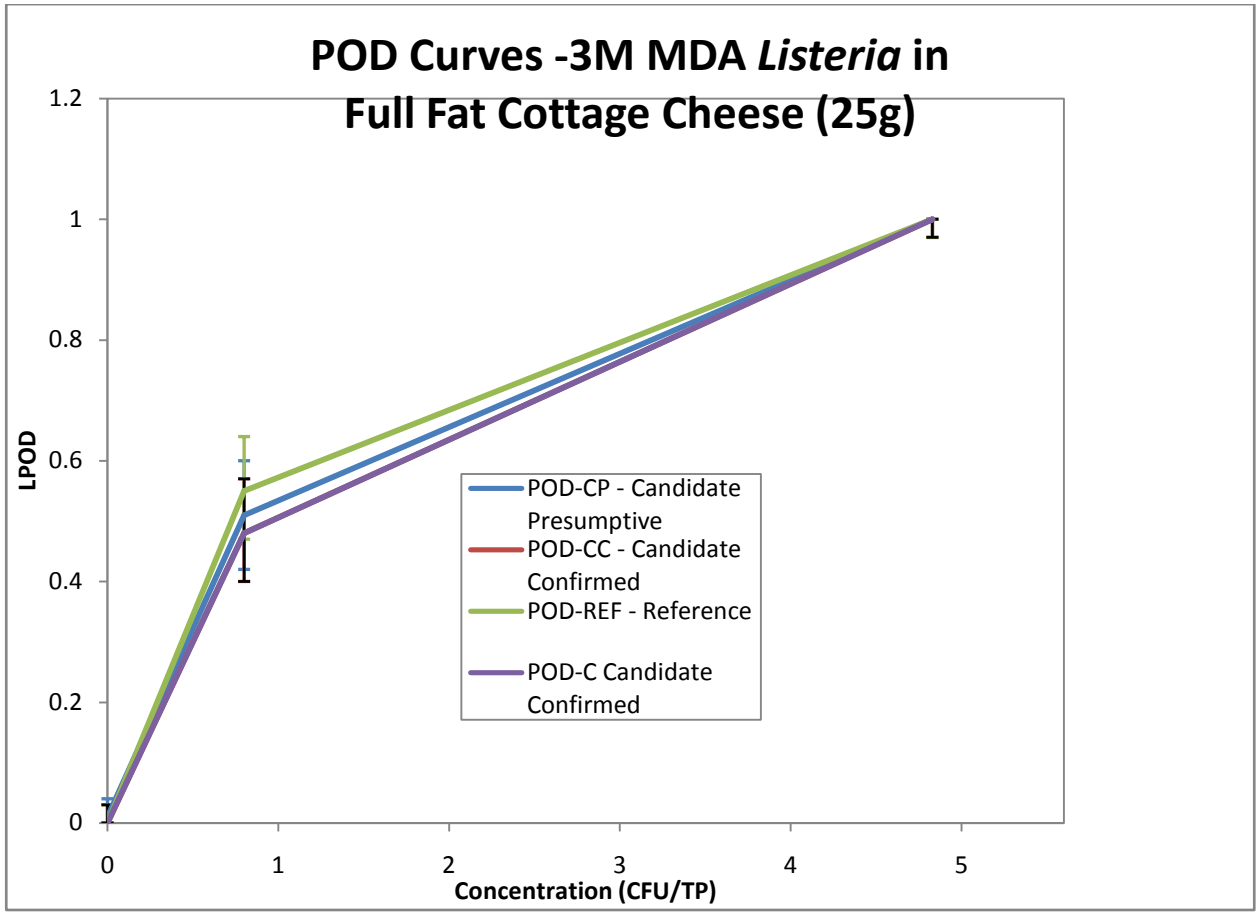
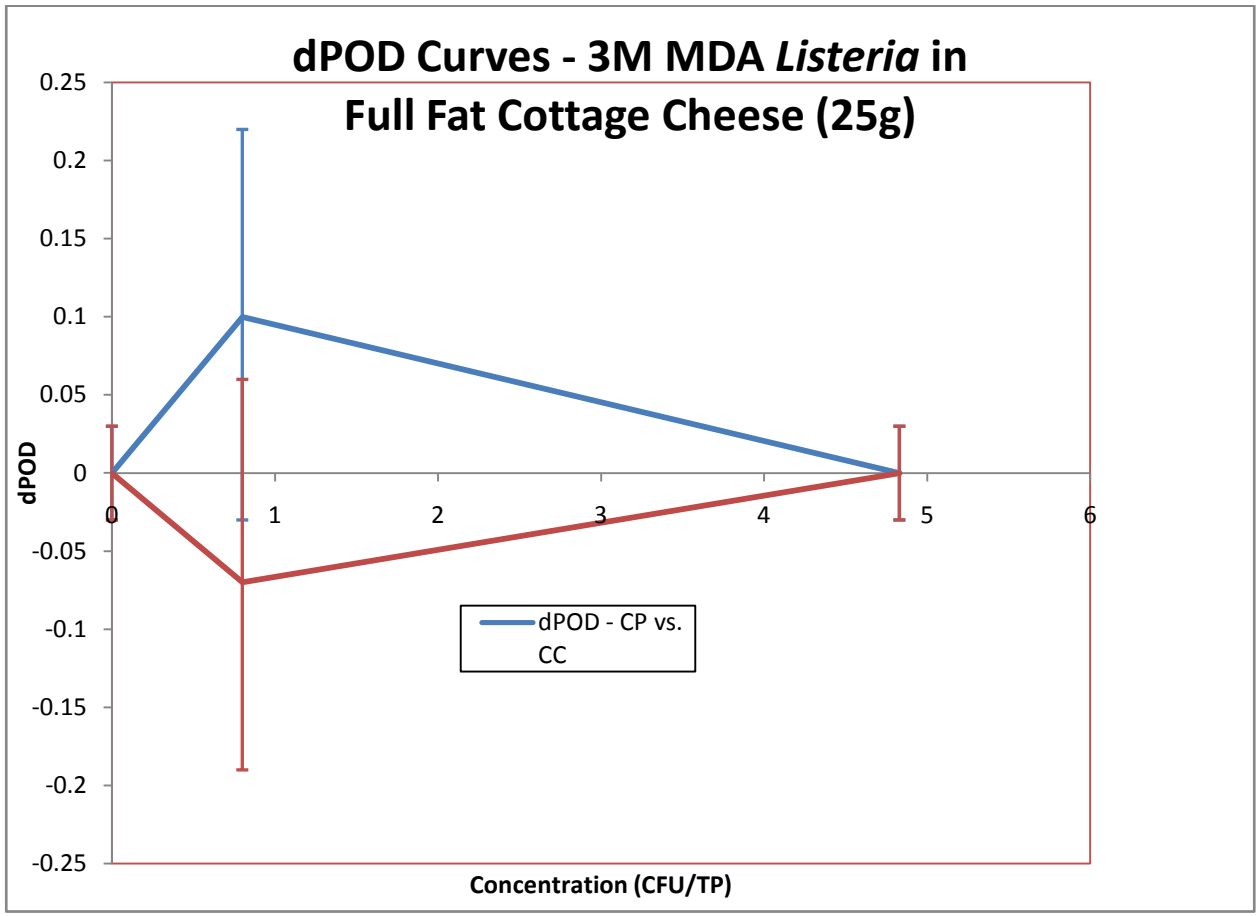


Figure 1B: dPOD Values of Candidate Method vs. Reference Method



AOAC Official Method 2014.07
***Listeria monocytogenes* in Selected Foods and Environmental Surfaces**
3M™ Molecular Detection Assay (MDA) *Listeria monocytogenes* Method
First Action 2014

[Applicable to detection of *Listeria monocytogenes* in beef hot dogs (25 and 125 g), deli turkey (25 and 125 g), cold smoked salmon (25 g), full-fat cottage cheese (25 g), chocolate milk (25 g), and two environmental surfaces: sealed concrete (sponge in 100 mL and sponge in 225 mL) and stainless steel (sponge in 225 mL).]

See Tables **2014.07A** and **2014.07B** for a summary of results of the interlaboratory study supporting acceptance of the method.

See Appendix available on the *J. AOAC Int*. website for supplementary materials for detailed results of the interlaboratory study (http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac).

A. Principle

The 3M™ Molecular Detection Assay (MDA) *Listeria monocytogenes* is intended for use with the 3M Molecular Detection System for the rapid and specific detection of *Listeria monocytogenes* in selected foods and environmental surfaces. The 3M MDA uses loop-mediated isothermal amplification to rapidly amplify nucleic acid sequences with high specificity and sensitivity, combined with bioluminescence to detect the amplification. Presumptive positive results are reported in real-time, while negative results are displayed after the assay is completed. Samples are enriched in prewarmed Demi-Fraser (DF) broth base, which does not contain ferric ammonium citrate (FAC).

B. Apparatus and Reagents

Items (**a**) and (**h**)-(**o**) are available from 3M Food Safety (St. Paul, MN, USA). Items (**b**)-(**g**) are available as the 3M MDA *Listeria monocytogenes* kit from 3M Food Safety.

(**a**) *3M™ Molecular Detection Instrument.*

(**b**) *3M MDA Listeria monocytogenes reagent tubes.*--Twelve strips of eight tubes.

(**c**) *Lysis solution (LS) tubes.*–Twelve strips of eight tubes.

(**d**) *Extra caps.*–Twelve strips of eight caps.

(**e**) *Negative control (NC).*--One vial (2 mL).

(**f**) *Reagent control (RC).*–Two pouches. Each pouch contains eight reagent tubes.

(**g**) *Quick Start Guide.*

(**h**) *3M™ Molecular Detection Speed Loader Tray.*

(**i**) *3M™ Molecular Detection Chill Block Tray and Chill Block Insert.*

(**j**) *3M™ Molecular Detection Heat Block Insert.*

(**k**) *3M™ Molecular Detection Cap/Decap Tool-Reagent (for reagent tubes).*

(**l**) *3M™ Molecular Detection Cap/Decap Tool-Lysis (for lysis tubes).*

(**m**) *Empty lysis tube rack.*

(**n**) *Empty reagent tube rack.*

(**o**) *DF broth base.*–Formulation equivalent to ISO 11290-1.

(**p**) *Disposable pipet.*–Capable of 20 µL.

(**q**) *Multichannel (8-channel) pipet.*--Capable of 20 µL.

(**r**) *Sterile filter tip pipet tips.*--Capable of 20 µL.

(**s**) *Filter Stomacher® bags.*–Seward or equivalent.

(**t**) *Stomacher.*–Seward or equivalent.

(**u**) *Thermometer.*–Calibrated range to include 100 ± 1°C.

(**v**) *Dry double block heater unit or water bath.*–Capable of maintaining 100 ± 1°C.

(**w**) *Incubators.*–Capable of maintaining 37 ± 1°C.

(**x**) *Freezer.*–Capable of maintaining -10 to -20°C, for storing the 3M Molecular Detection Chill Block Tray.

(**y**) *Refrigerator.*–Capable of maintaining 2-8°C, for storing the 3M MDA.

(**z**) *Computer.*–Compatible with the 3M Molecular Detection Instrument.

C. General Instructions

(**a**) Store the 3M MDA *Listeria monocytogenes* at 2-8°C. Do not freeze. Keep kit away from light during storage. After opening the kit, check that the foil pouch is undamaged. If the pouch is damaged, do not use. After opening, unused reagent tubes should always be stored in the resealable pouch with the desiccant inside to maintain stability of the lyophilized reagents. Store resealed pouches at 2-8°C for no longer than 1 month. Do not use 3M MDA *Listeria monocytogenes* past the expiration date.

(**b**) The 3M Molecular Detection Instrument is intended for use with samples that have undergone heat treatment during the assay lysis step, which is designed to destroy organisms present in the sample. Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should *not* be inserted into the 3M Molecular Detection Instrument.

(**c**) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

D. Safety Precautions

After use, the enrichment medium and the 3M MDA *Listeria monocytogenes* tubes can potentially contain pathogenic materials. *Listeria monocytogenes* is of particular concern for pregnant women, the aged, and the infirmed. It is recommended that these groups of concern avoid handling this organism. When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet (MSDS) for additional information and local regulations for disposal.

Periodically decontaminate laboratory benches and equipment (pipets, cap/decap tools, etc.) with a 1-5% (v/v in water) household bleach solution or DNA removal solution.

E. Sample Enrichment

(**a**) Prewarm DF broth base without FAC to 37 ± 1°C.

(**b**) Aseptically combine the enrichment medium and sample following the procedures in Table **2014.07C**. For all meat and highly particulate samples, the use of filter bags is recommended. Homogenize thoroughly for 2 ± 0.5 min. Incubate at 37 ± 1°C.

F. Preparation of the 3M Molecular Detection Speed Loader Tray

(**a**) Wet a cloth or paper towel with a 1-5% (v/v in water) household bleach solution and wipe the 3M Molecular Detection Speed Loader Tray.

(**b**) Rinse the 3M Molecular Detection Speed Loader Tray with water.

(**c**) Use a disposable towel to wipe the 3M Molecular Detection Speed Loader Tray dry.

(**d**) Ensure the 3M Molecular Detection Speed Loader Tray is dry before use.

G. Preparation of the 3M Molecular Detection Chill Block Insert

Before using the 3M Molecular Detection Chill Block Insert, ensure it has been stored on the 3M Molecular Detection Chill Block Tray in the freezer (-10 to -20°C) for a minimum of 2 h before use. When removing the 3M Molecular Detection Chill Block Insert from the freezer for use, remove it and the 3M Molecular Detection Chill Block Tray together. Use the 3M Molecular Detection Chill Block Insert/3M Molecular Detection Chill Block Tray within 20 min.

H. Preparation of the 3M Molecular Detection Heat Block Insert

Place the 3M Molecular Detection Heat Block Insert in a dry double block heater unit. Turn on the dry block heater unit and set the temperature to allow the 3M Molecular Detection Heat Block Insert to reach and maintain a temperature of 100 ± 1°C.

Note: Depending on the heater unit, allow approximately 30-50 min for the 3M Molecular Detection Heat Block Insert to reach temperature. Using a calibrated thermometer, verify that the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C.

I. Preparation of the 3M Molecular Detection Instrument

(**a**) Launch the 3M Molecular Detection Software and log in.

(**b**) Turn on the 3M Molecular Detection Instrument.

(**c**) Create or edit a run with data for each sample. Refer to the 3M Molecular Detection System User Manual for details.

Note: The 3M Molecular Detection Instrument must reach and maintain temperature of 60°C before inserting the 3M Molecular Detection Speed Loader Tray with reaction tubes. This heating step takes approximately 20 min and is indicated by an orange light on the instrument's status bar. When the instrument is ready to start a run, the status bar will turn green.

J. Lysis

(**a**) Allow the LS tubes to warm up to room temperature (20-25°C) by setting the rack on the laboratory bench for 2 h. *Alternatives to equilibrate the LS tubes to room temperature are to incubate the LS tubes in a 37 ± 1°C incubator for 1 h or at room temperature overnight (16-18 h).*

(**b**) Remove the enrichment broth from the incubator and gently agitate the contents.

(**c**) One LS tube is required for each sample and the NC sample.

(*1*) LS tube strips can be cut to desired LS tube number. Select the number of individual LS tubes or 8-tube strips needed. Place the LS tubes in an empty rack.

(*2*) To avoid cross-contamination, decap one LS tube strip at a time and use a new pipet tip for each transfer step.

(**d**) Transfer enriched sample to LS tubes as described below:

Note: Transfer each enriched sample into individual LS tubes first. Transfer the NC last.

(*1*) Use the 3M Molecular Detection Cap/Decap Tool-Lysis to decap one LS tube strip, one strip at a time. Set the tool with cap attached aside on a clean surface.

(*2*) Transfer 20 µL of sample into an LS tube.

(*3*) Repeat step (**d**)(*2*) until each individual sample has been added to a corresponding LS tube in the strip.

(*4*) Use the 3M Molecular Detection Cap/Decap Tool-Lysis to recap the LS tube strip. Use the rounded side of the tool to apply pressure in a back-and-forth motion ensuring that the cap is tightly applied. *See* Figure **2014.07A**.

(*5*) Repeat steps (**d**)(*1*)-(**d**)(*4*) as needed, for the number of samples to be tested.

(*6*) When all samples have been transferred, then transfer 20 µL NC into an LS tube. Use the 3M Molecular Detection Cap/Decap Tool-Lysis tool to recap the LS tube.

(*7*) Cover the rack of LS tubes with the rack lid and firmly invert 3-5 times to mix. *Suspension has to flow freely inside the tube.*

(**e**) Verify that the temperature of the 3M Molecular Detection Heat Block Insert is at 100 ± 1°C. Place the rack of LS tubes in the 3M Molecular Detection Heat Block Insert and heat for 15 ± 1 min. *An alternative to using dry heat for the lysis step is to use a water bath at 100 ± 1°C. Ensure that sufficient water is used to cover up to the liquid level in the LS tubes. Place the rack of LS tubes in the water bath at 100 ± 1°C and heat for 15 ± 1 min.* Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should *not* be inserted into the 3M Molecular Detection Instrument.

(**f**) Remove the rack of LS tubes from the heating block and allow to cool in the 3M Molecular Detection Chill Block Insert for 10 ± 1 min. *The LS solution may freeze when processing less than 48 LS tubes. Freezing of the LS solution will not affect the test. If freezing is observed, allow the LS tubes to thaw for 5 min before mixing. Remove the rack lid during incubation on the 3M Molecular Detection Chill Block Insert.*

(**g**) Remove the rack of LS tubes from the 3M Molecular Detection Chill Block Insert/3M Molecular Detection Chill Block Tray system. Replace the lid on the rack of LS tubes and firmly invert 3-5 times to mix. *Suspension has to flow freely inside the tube.*

(**h**) Firmly tap the lysis tubes rack on the laboratory bench 3-5 times.

(**i**) Place the rack on the laboratory bench and let sit undisturbed for 5-10 min to allow the resin to settle. *Do not mix or disturb the resin at the bottom of the tube. See* Figure **2014.07B**.

K. Amplification

(**a**) One reagent tube is required for each sample and the NC.

(*1*) Reagent tube strips can be cut to desired tube number. Select the number of individual reagent tubes or 8-tube strips needed.

(*2*) Place reagent tubes in an empty rack.

(*3*) Avoid disturbing the reagent pellets from the bottom of the tubes.

(**b**) Select one RC tube and place in rack.

(**c**) To avoid cross-contamination, decap one reagent tube strip at a time and use a new pipet tip for each transfer step.

(**d**) Transfer lysate to reagent tubes and RC tube as described below:

Note: Transfer each sample lysate into individual reagent tubes first followed by the NC. Hydrate the RC tube last.

Warning: Care must be taken when pipetting LS, as carry-over of the resin may interfere with amplification.

(*1*) Use the 3M Molecular Detection Cap/Decap Tool-Reagent to decap the reagent tubes, one reagent tube strip at a time. Discard cap.

(*2*) Transfer 20 µL of sample lysate from the upper portion of the fluid in the LS tube into corresponding reagent tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down 5 times.

(*3*) Repeat step (**d**)(*2*) until individual sample lysate has been added to a corresponding reagent tube in the strip.

(*4*) Cover the reagent tubes with the provided extra cap and use the rounded side of the 3M Molecular Detection Cap/Decap Tool-Reagent to apply pressure in a back and forth motion ensuring that the cap is tightly applied.

(*5*) Repeat steps (**d**)(*1*)-(**d**)(*4*) as needed, for the number of samples to be tested.

(*6*) When all sample lysates have been transferred, repeat steps (**d**)(*1*)-(**d**)(*4*) to transfer 20 µL NC lysate into a reagent tube.

(*7*) Transfer 20 µL NC lysate into an RC tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down 5 times.

(**e**) Load capped tubes into a clean and decontaminated 3M Molecular Detection Speed Loader Tray. Close and latch the 3M Molecular Detection Speed Loader Tray lid (Figure **2014.07C**).

(**f**) Review and confirm the configured run in the 3M Molecular Detection Software.

(**g**) Click the Start button in the software and select instrument for use. The selected instrument's lid automatically opens.

(**h**) Place the 3M Molecular Detection Speed Loader Tray into the 3M Molecular Detection Instrument and close the lid to start the assay. Results are provided within 75 min, although positives may be detected sooner.

(**i**) After the assay is complete, remove the 3M Molecular Detection Speed Loader Tray from the 3M Molecular Detection Instrument and dispose of the tubes by soaking in a 1-5% (v/v in water) household bleach solution for 1 h and away from the assay preparation area.

Notice: To minimize the risk of false positives due to cross-contamination, never open reagent tubes containing amplified DNA. This includes RC, reagent, and Matrix Control tubes. Always dispose of sealed reagent tubes by soaking in a 1-5% (v/v in water) household bleach solution for 1 h and away from the assay preparation area.

L. Results and Interpretation

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A Positive or Negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real-time while Negative and Inspect results will be displayed after the run is completed. Presumptive positive results should be confirmed using one's preferred method or as specified by the FDA/BAM, USDA/FSIS-MLG, AOAC *Official Method*^{SM} **993.12**, or ISO 11290 methods starting from the 3M primary enrichment, followed by secondary enrichment or direct plating and confirmation of isolates using appropriate biochemical and serological methods.

Note: Even a negative sample will not give a zero reading as the system and 3M Molecular Assay *Listeria monocytogenes* amplification reagents have a "background" relative light unit (RLU).

In the rare event of any unusual light output, the algorithm labels this as "Inspect." 3M recommends the user to repeat the assay for any Inspect samples. If the result continues to be Inspect, proceed to confirmation test using one's preferred method or as specified by local regulations.

Reference: *J. AOAC Int*. (future issue)

Posted: January 29, 2015

Table 2014.07A. POD summary of full-fat cottage cheese results for the 3M MDA *Listeria monocytogenes*

Method[a]	3M™ MDA™ *Listeria monocytogenes*		
Inoculation level	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	0/132	66/132	129/132
Candidate presumptive POD (CP)	0.00 (0.00, 0.03)	0.50 (0.41, 0.59)	0.98 (0.94, 0.99)
s_r[b]	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.15 (0.13, 0.17)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.17)	0.03 (0.00, 0.08)
s_R[d]	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.15 (0.13, 0.18)
P-value[e]	1.000	0.9123	0.9499
Candidate confirmed positive/total No. of samples analyzed	0/132	64/132	132/132
Candidate confirmed POD (CC)	0.00 (0.00, 0.03)	0.48 (0.40, 0.57)	1.00 (0.97, 1.00)
s_r	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.16)	0.00 (0.00, 0.15)	0.00 (0.00, 0.16)
s_R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P-value	1.0000	0.8762	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/132	63/132	129/132
Candidate confirmed POD (C)	0.00 (0.00, 0.03)	0.48 (0.39, 0.57)	0.98 (0.94, 0.99)
s_r	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.15 (0.13, 0.17)
s_L	0.00 (0.00, 0.16)	0.00 (0.00, 0.14)	0.03 (0.00, 0.08)
s_R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.15 (0.13, 0.18)
P-value	1.0000	0.9080	0.9499
Positive reference samples/total No. of samples analyzed	0/132	73/132	132/132
Reference POD	0.00 (0.00, 0.03)	0.55 (0.47, 0.64)	1.00 (0.97, 1.00)
s_r	0.00 (0.00, 0.16)	0.50 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.16)	0.00 (0.00, 0.18)	0.00 (0.00, 0.16)
s_R	0.00 (0.00, 0.23)	0.50 (0.45, 0.52)	0.00 (0.00, 0.23)
P-value	1.0000	0.6678	1.0000
dLPOD (candidate vs reference)[f]	0.00 (-0.03, 0.03)	-0.08 (-0.20, 0.05)	-0.02 (-0.06, 0.01)
dLPOD (candidate presumptive vs candidate confirmed)[f]	0.00 (-0.03, 0.03)	0.02 (-0.11, 0.14)	-0.02 (-0.06, 0.01)

[a] Results include 95% confidence intervals.

[b] s_r = Repeatability standard deviation.

[c] s_L = Among-laboratory standard deviation.

[d] s_R = Reproducibility standard deviation.

[e] *P*-value = Homogeneity test of laboratory PODs.

[f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods.

Table 2014.07B. POD summary of deli turkey results for the 3M MDA *Listeria monocytogenes*

Method[a]	3M™ MDA™ *Listeria monocytogenes*		
Inoculation level	Uninoculated	Low	High
Candidate presumptive positive/total No. of samples analyzed	2/132	66/132	132/132
Candidate presumptive POD (CP)	0.02 (0.01, 0.05)	0.50 (0.41, 0.59)	1.00 (0.97, 1.00)
s_r[b]	0.12 (0.11, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.05)	0.00 (0.00, 0.17)	0.00 (0.00, 0.16)
s_R[d]	0.12 (0.11, 0.14)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P-value[e]	0.5190	0.7565	1.000
Candidate confirmed positive/total No. of samples analyzed	1/132	67/132	132/132
Candidate confirmed POD (CC)	0.01 (0.00, 0.04)	0.51 (0.42, 0.60)	1.00 (0.97, 1.00)
s_r	0.09 (0.08, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.04)	0.00 (0.00, 0.17)	0.00 (0.00, 0.16)
s_R	0.09 (0.08, 0.10)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P-value	0.4338	0.7891	1.0000
Candidate confirmed positive/total No. of samples analyzed	0/132	64/132	132/132
Candidate confirmed POD (C)	0.00 (0.00, 0.03)	0.48 (0.40, 0.57)	1.00 (0.97, 1.00)
s_r	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)
s_R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P-value	1.0000	0.8249	1.000
Positive reference samples/total No. of samples analyzed	0/132	66/132	132/132
Reference POD	0.00 (0.00, 0.03)	0.50 (0.41, 0.59)	1.00 (0.97, 1.00)
s_r	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L	0.00 (0.00, 0.16)	0.00 (0.00, 0.17)	0.00 (0.00, 0.16)
s_R	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P-value	1.0000	0.7565	1.0000
dLPOD (candidate vs reference)[f]	0.00 (-0.03, 0.03)	-0.02 (-0.14, 0.11)	0.00 (-0.03, 0.03)
dLPOD (candidate presumptive vs candidate confirmed)[f]	0.01 (-0.03, 0.05)	-0.01 (-0.13, 0.12)	0.00 (-0.03, 0.03)

[a] Results include 95% confidence intervals.

[b] s_r = Repeatability standard deviation.

[c] s_L = Among-laboratory standard deviation.

[d] s_R = Reproducibility standard deviation.

[e] *P*-value = Homogeneity test of laboratory PODs.

[f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods.

Table 2014.07C. Enrichment protocols for the 3M MDA *Listeria monocytogenes*[a]

Sample matrix	Sample size	Primary enrichment, Demi-Fraser broth (no FAC)		
		Enrichment broth volume, mL	Enrichment temperature (±1°C)	Enrichment time, h
Food				
Full-fat cottage cheese	25 g	225	37	24-28
Chocolate milk	25 g	225	37	24-28
Beef hot dogs	25 g	225	37	26-30
	125 g	1125	37	26-30
Deli turkey	25 g	225	37	26-30
	125 g	1125	37	26-30
Cold smoked salmon	25 g	225	37	26-30
Environmental surfaces				
Sealed concrete	1 Sponge	100	37	26-30
Sealed concrete, stainless steel	1 Sponge	225	37	26-30

[a] A 24-26 h primary enrichment in Demi-Fraser broth base (no FAC), followed by a 24-26 h secondary enrichment in Fraser broth base (no FAC), is also acceptable for these matrices.

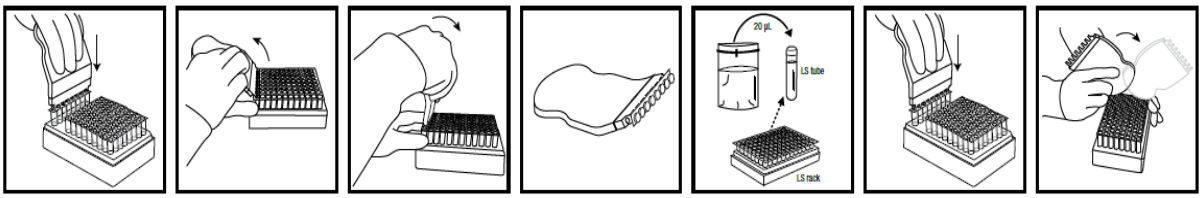


Figure 2014.07A. Transfer of enriched sample to lysis solution tube.

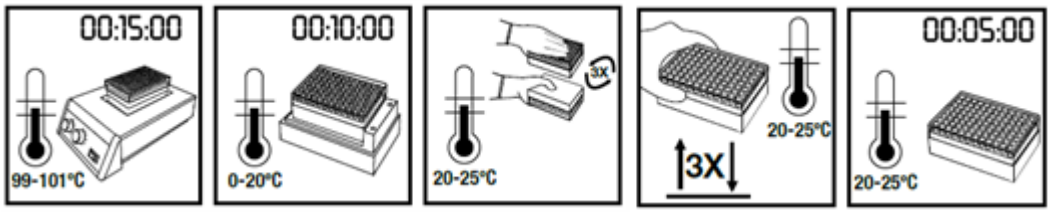


Figure 2014.07B. Sample lysis.

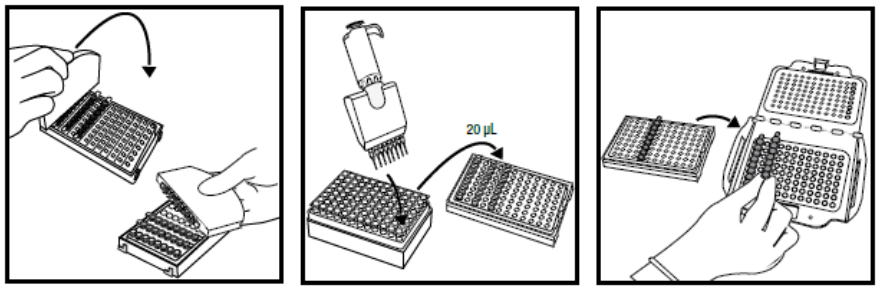


Figure 2014.07C. Transfer of lysate to reagent tube.

Evaluation of 3M™ Molecular Detection Assay (MDA) *Listeria monocytogenes* for the Detection of *Listeria monocytogenes* in Selected Foods and Environmental Surfaces: Collaborative Study

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The 3M™ Molecular Detection Assay (MDA) *Listeria monocytogenes*combines isothermal amplification and bioluminescence to detect *Listeria monocytogenes*with high specificity and efficiency in select foods and environmental samples. The 3M MDA *Listeria monocytogenes*method was evaluatedusing an unpaired study design in a multi-laboratory collaborative study to the USDA/FSIS MLG 8.09 (2011) *Isolation and Identification of Listeria monocytogenes fromRed Meat, Poultry, and Egg Products and Environmental Samples* fordeli turkeyand the AOAC® Official Method of Analysis 993.12 *Listeria monocytogenes in Milk and Dairy Products* for full fat (4% milk fat) cottage cheese following the current AOAC Guidelines. A total of 16 laboratories located in the continental United States and Canada participated in this collaborative study. For deli turkey, 125 g test portions were evaluated using heat stressed cells by each method. Forfull fat cottage cheese, 25g test portions were evaluated using non-heat stressed cells. Each matrix had 3 inoculation levels: an un-inoculated control level (0 colony forming units (CFU)/test portion), and two levels artificially contaminated with *Listeria monocytogenes,* a low inoculum level (0.2-2 CFU/test portion) and a high inoculum level (2-5 CFU/test portion).In total, 1,584 unpaired replicate samples were analyzed. Statistical analysis was conducted according to the Probability of Detection (POD). Results obtained for the low inoculum level full fat cottage cheese test portions produced a dLPOD value of -0.08, (-0.20, 0.05) with 95% Confidence Intervals.For the low-level deli turkey test portions, a dLPOD value of -0.02, (-0.14, 0.11)with 95% Confidence Intervals was obtained.

Listeria monocytogenes, a Gram positive rod shaped facultative bacterium, infects roughly 1,600 persons annually [1, 2]. On average, infections caused by *Listeria monocytogenes* result in 255 deaths in the United States annually producing one of the highest mortality rates for a foodborne pathogen [2]. With its ability to survive and grow in various harsh environments including

propagation at temperatures lower than 1°C, *Listeria monocytogenes*continues to be a nuisance to the food industry [2].

The 3M MDA *Listeria monocytogenes* method allows for the rapid and specific detection of*Listeria monocytogenes*in food and environmental samplesafter 24 hours of enrichment using pre-warmed (37 ± 1°C) Demi-Fraser (DF) broth base (without ferric ammonium citrate (FAC)).After enrichment, samples are evaluated using the 3M™ MDA *Listeria monocytogenes* on the 3M™ Molecular Detection System (MDS). Presumptive positive results are reported in real-time while negative results are displayed after completion of the assay(75 minutes).

Prior to the collaborative study, the 3M MDA *Listeria monocytogenes* method was certified as an AOAC® *Performance Tested*℠ *Method* (PTM) following the AOAC Guidelines for Harmonized PTM Studies [3]. The goal of the PTM study was to demonstrate that the 3M MDA *Listeria monocytogenes* method could detect *Listeria monocytogenes* in selected foods and environmental surfaces as claimed by the manufacturer. For the 3M MDA *Listeria monocytogenes*evaluation, there were 8 food matrices and 2 environmental surfaces analyzed: beef hot dogs (25 g & 125 g), deli turkey (25 g & 125 g), cold smoked salmon (25 g), full fat cottage cheese (25 g), chocolate milk (25 g), bagged raw spinach (25 g), romaine lettuce (25 g), cantaloupe (whole melon), sealed concrete (sponge in 100 mL & 225 mL) and stainless steel (sponge in 225 mL) usingDF broth basewithout FAC and where applicable, a secondary enrichment in Fraser broth base without FAC.All other PTM parameters (inclusivity, exclusivity, ruggedness, stability and lot to lot variability) tested in the PTM studies satisfied the performance requirements for PTM approval. The method was awarded PTM certification number 051401 on May 23, 2014.

The aim of this collaborative studywas to compare the reproducibility of the 3M MDA *Listeria monocytogenes* method to the United States Department of Agriculture (USDA)Food Safety Inspection Service (FSIS) -Microbiology Laboratory Guidebook (MLG) 8.09*Isolation and Identification of Listeria monocytogenes from Red Meat, Poultry, and Egg Products and Environmental Samples*[4] for deli turkey and the AOAC® Official Methodof Analysis 993.12*Listeria monocytogenes in Milk and Dairy Products* [5] reference method for full fat (4% milk fat) cottage cheese.

Collaborative Study

Study Design

In this collaborative study, two matrices, deli turkey and full fat cottage cheese, were evaluated. The matrices were obtained from a local retailer and screened for the absence of *Listeria monocytogenes*by the appropriate reference methods prior to analysis. The deli turkey was artificially contaminated with heat stressed cells of*Listeria monocytogenes*American Type Culture Collection (ATCC)13932 and the full fat cottage cheese with non-heat stressed cells of *Listeria monocytogenes*ATCC 19114. There were two inoculation levels for each matrix: a high inoculation level of approximately 2-5 colony-forming units (CFU)/test portion and a low inoculation level of approximately 0.2-2 CFU/test portion. A set of un-inoculated control test portions were also included for each matrix at 0 CFU/test portion for a total of three contamination levels per method.

Twelve replicate samples from each of the three contamination levels were analyzed. Two sets of samples (72 total) were sent to each laboratory for analysis by 3M MDA *Listeria monocytogenes* and either the USDA/FSIS- MLG (deli turkey) or AOAC OMA 993.12 (full fat cottage cheese) reference method due to different sample enrichment procedures between the

candidate method and the reference methods. Additionally, collaborators were sent a 30 g test portion and instructed to conduct a total aerobic plate count (APC) using 3M™ Petrifilm Aerobic Count Plate (AOAC Official Method 990.12) [6] on the day samples were received for the purpose of determining the total aerobic microbial load.

A detailed collaborative study packet outlining all necessary information related to the study including media preparation, test portion preparation and documentation of results was sent to each collaborating laboratory prior to the initiation of the study. A conference call was conducted to discuss the collaborative study packet and answer any questions from the participating laboratories.

Preparation of Inocula and Test Portions

The *Listeria monocytogenes*cultures used in this evaluation were propagated in 10 mL of Brain Heart Infusion (BHI) broth from a Q Laboratories frozen stock culture held at -70°C. Each organism was incubated for 18 ± 0.5 hours at 35 ±1°C. Prior to inoculation, the culture suspension for the deli turkey was heat stressed at 50 ± 1°C in a water bath for 10 ±0.5minutes to obtain a percent injury of 50-80% (as determined by plating onto selective modified Oxford agar (MOX) and non-selective Tryptic Soy agar with yeast extract (TSA/ye). The degree of injury was estimated as:

$$(1-\frac{n_{select}}{n_{nonselect}})x100$$

where n_{select} = number of colonies on selective agar and $n_{nonselect}$ = number of colonies on non-selective agar.Appropriate dilutions of each culture were prepared in Butterfields' Phosphate Diluent (BPD) based on previously established growth curves for both low and high inoculation levels. Bulk portions of each matrix were inoculated with the diluted liquid inoculum and mixed thoroughly by hand mixing to ensure an even distribution of microorganisms. The inoculated full fat cottage cheese was divided into separate 30 g portions packaged in sterile whirl-pak® bags and shipped to the collaborators. For the analysis of the deli turkey, 25 g of inoculated test product was mixed with 100g of un-inoculated test product to prepare 125 g test portions which were packaged in sterile whirl-pak® bags..

To determine the level of *Listeria monocytogenes* in the matrices, a 5-tube most probable number (MPN) was conducted by the coordinating laboratory on the day of initiation of analysis using the USDA/FSIS-MLG 8.09 reference method for deli turkey or the AOAC OMA 993.12 for the full fat cottage cheese. For deli turkey, the MPN of the high and low inoculated levels was determined by analyzing 5 x 250 g test portions, the reference method test portions from the collaborating laboratories and 5 x 60 g test portions by the USDA/FSIS-MLG 8.09 reference method. For the full fat cottage cheese, the MPN of the high and low inoculated levels was determined by analyzing5 x50g test portions, the reference method test portions from the collaborating laboratories and 5 x 10g test portions by the AOAC OMA 993.12 reference method. Each test portion was enriched at a 1:10 dilution and evaluated following the appropriate reference method. The MPN and 95% confidence intervals were calculated using the LCF MPN Calculator, Version 1.6, provided by AOAC RI(www.lcftld.com/customer/LCFMPNCaclucator.exe) [7].Confirmation of the samples was conducted according to the USDA/FSIS-MLG 8.09 or the AOAC OMA 993.12 reference method, dependent on the matrix.

Test Portion Distribution

All samples were labeled with a randomized, blind-coded 3 digit number affixed to the sample container. Test portions were shipped on a Thursday via overnight delivery according to the Category B Dangerous Goods shipment regulations set forth by the International Air Transportation Association(IATA). Upon receipt, samples were held by the collaborating laboratory at refrigeration temperature (3-5 °C) until the following Monday when analysis was initiated a total of 96 hours post-inoculation. All samples were packed with cold packs to target a temperature of < 7°C during shipment. In addition to each of the test portions and the total plate count replicate, collaborators also received a test portion for each matrix labeled as 'temperature control'. Participants were instructed to obtain the temperature of this portion upon receipt of the package, document results on the Sample Receipt Confirmation form provided and fax to the study director. The shipment and hold times (through 120 hours) of the inoculated test material had been verified as a quality control measure prior to study initiation.

Test Portion Analysis

Each collaborator received 72 test portions (12 high inoculum, 12 low inoculum and 12 uninoculated controls for each method) of each matrix. Collaborators followed the appropriate preparation and analysis protocol according to the method specified for the matrix.

For the analysis of the deli turkey test portions by the 3M MDA *Listeria monocytogenes* method, a 125 g portion was enriched with 1125 mL of pre-warmed (37 ±1°C) DF broth base without FAC, homogenized for 2 ± 0.5 minutes and incubated for 26-30 hours at 37 ±1°C. For the full fat cottage cheese test portions analyzed by the 3M MDA *Listeria monocytogenes* method, a 25 g portion was enriched with 225 mL pre-warmed (37 ±1°C)DF broth base without FAC, homogenized for 2 ± 0.5 minutes and incubated for 24-28 hours at 37 ±1°C. Following enrichment, samples were assayed by the 3M MDA *Listeria monocytogenes* method and confirmed following the standard reference method specified for each matrix.

Following enrichment, full fat cottage cheese samples assayed by the 3M MDA *Listeriamonocytogenes* method were confirmed by streaking an aliquot of the primary enrichment onto Oxford Agar (OXA). Presumptive positive samples were streaked for isolation on TSA/ye, verified morphologically by Gram stain and biochemically confirmed byhemolysis testing and by VITEK 2 GP Biochemical Identification method (AOAC OMA 2012.02) [8] or API Listeria Identification System biochemical test kits. Laboratories utilizing API Listeria kits were also required to conduct catalase and oxidase tests.

For samples analyzed using the AOAC OMA 993.12 reference method, 25 g test portions were enriched in pre-warmed (45°C) selective enrichment broth, homogenized for 2 ± 0.5 minutes and incubated at 30 ± 2°C for 48 hours. Samples were streaked onto OXA and presumptive positive samples were streaked for isolation onto TSA/ye. Colonies from TSA/ye were verified morphologically by Gram stain and biochemically confirmed by evaluation of a hemolytic reaction on sheep blood agar and biochemically confirmed VITEK 2 GP Biochemical Identification method or API Listeria biochemical test kits. Laboratories utilizing API Listeria kits were also required to conductcatalaseand oxidase tests.

Following enrichment, deli turkey samples assayed by the 3M MDA *Listeriamonocytogenes* method were confirmed by streaking an aliquot of the primary enrichment onto MOX and transferring an aliquot into Fraser Broth (FB). Presumptive positive samples were streaked for isolation on Horse Blood Overlay Agar (HL) and confirmed by evaluation of a hemolytic reaction and biochemically confirmedby the VITEK 2 GP Biochemical Identification methodor API Listeria Identification System biochemical test kits. Laboratories utilizing API Listeria kits were also required to conduct catalase and oxidase tests.

For samples analyzed using the USDA/ FSIS-MLG 8.09 reference method, 125g test portions were enriched with 1125 ± 25 mL of modified University of Vermont broth (UVM), homogenized for 2 ± 0.5 minutes and incubated for 24 hours at 30 ± 2°C. After incubation, samples were confirmed by streaking an aliquot of the primary enrichment onto MOX and transferring an aliquot into FB. Presumptive positive samples were streaked for isolation ontoHL and confirmed by evaluation of a hemolytic reaction and biochemically confirmed by the VITEK 2 GP Biochemical Identification methodor API Listeria Identification System biochemical test kits. Laboratories utilizing API Listeria kits were also required to conduct catalase and oxidase tests.

Statistical Analysis

Each collaborating laboratory recorded results for the reference method and the 3M MDA *Listeriamonocytogenes* method on the data sheets provided. The data sheets were submitted to the study director at the end of testing for analysis. The results of each test portion for each sample were compiled by the study director and the 3M MDA *Listeria monocytogenes* results were compared to the reference methods for statistical analysis. Data for each test portion size was analyzed using the probability of detection (POD) [9]. The probability of detection (POD) is the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent.The POD was calculated for the candidate presumptive results, $POD_{CP,}$ the candidate confirmatory results (including false negative results), POD_{CC}, the difference in the candidate presumptive and confirmatory results, $dPOD_{CP,}$ presumptive candidate results that confirmed positive (excluding false negative results), $POD_{C,}$ the reference method, POD_R, and the difference in the confirmed candidate and reference methods, $dPOD_C$. A dLPOD confidence interval not containing the point zero would indicate a statistically significant difference between the 3M MDA *Listeria monocytogenes* and the AOAC OMA 993.12 reference methods at the 5 % probability level. In addition to POD, the repeatability standard deviation (s_r), the among laboratory repeatibilty standard deviation (s_L), the reproducibility standard deviation ($s_{R)}$ and the P_T value were calculated. The s_r provides thevariance of data within one laboratory, the s_L provides the difference in standard deviation between laboratories and the s_R provides the variance in data between different laboratories. The P_T value provides information on thehomogeneity test of laboraotory PODs[10].

3M™ Molecular Detection Assay (MDA) *Listeria monocytogenes* Method
First Action 2014

Applicable to detection of *Listeria monocytogenes* in (beef hot dogs (25 g & 125 g), deli turkey (25 g & 125 g), cold smoked salmon (25 g), full fat cottage cheese (25 g), chocolate milk (25 g), and two environmental surfaces, sealed concrete (sponge in 100 mL;sponge in 225 mL) and stainless steel (sponge in 225 mL).

*See Table **2014.1A-B**for a summary of results of the inter-laboratory study.*
*See Tables **2014.2A-B** of the Supplementary Materialsfor detailed of results of the inter-laboratory study*

A. Principle

3M™ Molecular Detection Assay (MDA) *Listeria monocytogenes*method is intended for use with the 3M™ Molecular Detection System for the rapid and specific detection of *Listeriamonocytogenes* in selected food and environmental surfaces. The 3M MDA *Listeria monocytogenes* test uses isothermal amplification of unique DNA target sequences with high specificity, efficiency, rapidity and bioluminescence to detect the amplified sequences. Presumptive positive results are reported in real-time while negative results are displayed after the assay is completed. Samples areenriched in pre-warmed Demi Fraser (DF) broth base, which does not contain Ferric Ammonium Citrate (FAC).

B. Apparatus and Reagents

Items (b)-(g) are available as the 3M™ Molecular Detection Assay (MDA) *Listeria monocytogenes* kit from 3M Food Safety (St. Paul, MN 55144-1000, USA).

- **(a)** *3M Molecular Detection System* – Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
- **(b)** *3M Molecular Detection Assay Listeria monocytogenes reagent tubes*- 12 strips of 8 tubes. Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
- **(c)** *Lysis Solution (LS) tubes* – 12 strips of 8 tubes.
- **(d)** *Extra caps* – 12 strips of 8 caps
- **(e)** *Negative control (NC)* - One vial (2 mL).
- **(f)** *Reagent Control* – 8 reagent tubes
- **(g)** *Quick Start Guide*
- **(h)** *3M™ Molecular Detection Speed Loader Tray* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
- **(i)** *3M™ Molecular Detection Chill Block Tray and Chill block insert* - Available from 3M™ Food Safety (St. Paul, MN 55144-1000, USA).
- **(j)** *3M™ Molecular Detection Heat Block Insert* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
- **(k)** *3M™ Molecular Detection Cap/Decap Tool for Reagent tubes* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
- **(l)** *3M™ Molecular Detection Cap/Decap Tool for Lysis tubes* – Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
- **(m)***Empty lysis tube rack* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).

(n) *Empty reagent tube rack* - Available from 3M Food Safety (St. Paul, MN 55144-1000, USA).
(o) *Demi-Fraser Broth Base (DF)*– Available from 3M Food Safety (St. Paul, MN 55144-1000, USA). (Formulation equivalent to ISO 11290-1)
(p) *Disposable pipette* – capable of 20 µL
(q) *Multi*-channel (8-channel) pipette - capable of 20 µL
(r) *Sterile filter tip pipette tips* - capable of 20 µL
(s) *Filter Stomacher® bags* – Seward or equivalent.
(t) *Stomacher®*– Seward or equivalent.
(u) *Thermometer* – calibrated range to include 100 ± 1°C
(v) *Dry double block heater unit or water bath* – capable of maintaining 100 ± 1°C
(w) *Incubators.* – Capable of maintaining 37 ± 1°C
(x) *Freezer* – capable of maintaining -10 to -20 °C, for storing the 3M Molecular Detection Chill Block Tray
(y) *Refrigerator* – capable of maintaining 2-8°C, for storing the 3M Molecular Detection Assay
(z) *Computer* – compatible with the 3M™ Molecular Detection Instrument

C. General Instructions

(a) Store the 3M Molecular Detection Assay *Listeria monocytogenes* at 2-8°C. Do not freeze. Keep kit away from light during storage. After opening the kit, check that the foil pouch is undamaged. If the pouch is damaged, do not use. After opening, unused reagent tubes should always be stored in the re-sealable pouch with the desiccant inside to maintain stability of the lyophilized reagents. Store resealed pouches at 2-8°C for no longer than 1 month. Do not use 3M Molecular Detection Assay *Listeria monocytogenes* past the expiration date.

(b) The 3M Molecular Detection Instrument is intended for use with samples that have undergone heat treatment during the assay lysis step, which is designed to destroy organisms present in the sample. Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should NOT be inserted into the 3M Molecular Detection Instrument.

(c) Follow all instructions carefully. Failure to do so may lead to inaccurate results.

Safety Precautions

After use, the enrichment medium and the 3M Molecular Detection Assay *Listeria monocytogenes* tubes can potentially contain pathogenic materials. *Listeria monocytogenes* is of particular concern for pregnant women, the aged and the infirmed. It is recommended that these concerned groups avoid handling this organism.When testing is complete, follow current industry standards for the disposal of contaminated waste. Consult the Material Safety Data Sheet for additional information and local regulations for disposal.

Periodically decontaminate laboratory benches and equipment (pipettes, cap/decap tools, etc…) with a 1- 5% (v:v in water) household bleach solution or DNA removal solution.

D. Sample Enrichment

(a) Pre-warm DF broth base without FACat 37 ±1°C..
(b) Aseptically combine the enrichment medium and sample following the table below. For all meat and highly particulate samples, the use of filter bags is recommended. Homogenize thoroughly for 2 ± 0.5 minutes Incubate at 37 ±1°C.

Table A: Enrichment Protocols for the **3M MDA *Listeria monocytogenes***

	Sample Matrix		Sample Size	Primary Enrichment Demi-Fraser Broth (no FAC)					
				Enrichment Broth Volume (mL)	Enrichment Temperature (±1°C)	Enrichment Time (hr)			
	Full fat cottage cheese		25 g	225	37	24-28			
	Chocolate milk		25 g	225	37	24-28			
	Beef hot dogs		25 g	225	37	26-30			
			125 g	1125					
	Deli turkey		25 g	225	37	26-30			
			125 g	1125					
	Cold smoked salmon		25 g	225	37	26-30			
	Environmental surfaces	Sealed Concrete	1 sponge	100	37	26-30			
		Sealed Concrete, Stainless Steel	1 sponge	225					

*A 24-26 hr primary enrichment in Demi-Fraser Broth Base (no FAC) followed by a 24-26 hr secondary enrichment in Fraser Broth Base (no FAC) is also acceptable for these matrices.

E. Preparation of the 3M™ Molecular Detection Speed Loader Tray

(a) Wet a cloth or paper towel with a 1-5% (v:v in water) household bleach solution and wipe the 3M™ Molecular Detection Speed Loader Tray.
(b) Rinse the 3M Molecular Detection Speed Loader Tray with water.
(c) Use a disposable towel to wipe the 3M Molecular Detection Speed Loader Tray dry.

(d) Ensure the 3M Molecular Detection Speed Loader Tray is dry before use

F. PREPARATION OF THE 3M™ MOLECULAR DETECTIONCHILL BLOCK INSERT

Before using the 3M™ Molecular Detection Chill Block Insert, ensure it has been stored on the 3M™ Molecular Detection Chill Block Tray in the freezer (-10 to -20°C) for a minimum of 2 hours before use. When removing the 3M Molecular Detection Chill Block Insert from the freezer for use, remove it and the 3M Molecular Detection Chill Block Tray together. Use the 3M Molecular Detection Chill Block Insert /3M Molecular Detection Chill Block Tray within 20 minutes.

G. PREPARATION OF THE 3M™ MOLECULAR DETECTION HEAT BLOCK INSERT

Place the 3M™ Molecular Detection Heat Block Insert in a dry double block heater unit. Turn on the dry block heater unit and set the temperature to allow the 3M Molecular Detection Heat Block Insert to reach and maintain a temperature of 100 ±1°C.

NOTE: Depending on the heater unit, allow approximately 30-50 minutes for the 3M Molecular Detection Heat Block Insert to reach temperature. Using a calibrated thermometer, verify that the 3M Molecular Detection Heat Block Insert is at 100 ±1°C.

H. PREPARATION OF THE 3M MOLECULAR DETECTION INSTRUMENT

(a) Launch the 3M™ Molecular Detection Software and log in.
(b) Turn on the 3M Molecular Detection Instrument.
(c) Create or edit a run with data for each sample. Refer to the 3M Molecular Detection System User Manual for details.

NOTE: The 3M Molecular Detection Instrument must reach and maintain temperature of 60°C before inserting the 3M Molecular Detection Speed Loader Tray with reaction tubes. This heating step takes approximately 20 minutes and is indicated by an ORANGE light on the instrument's status bar. When the instrument is ready to start a run, the status bar will turn GREEN.

I. LYSIS

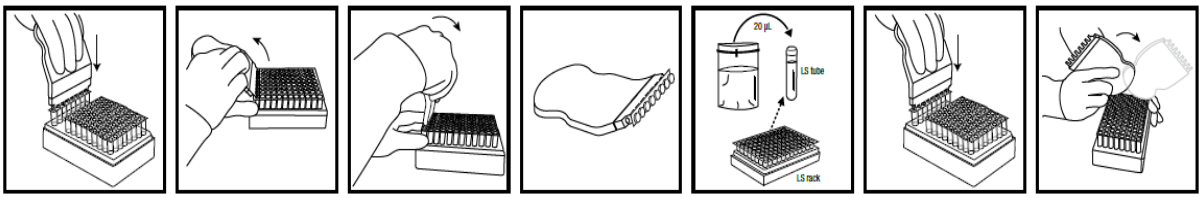
1. Allow the lysis solution(LS) tubes to warm up to room temperature by setting the rack on the laboratory bench for 2 hours [(a)].
2. Remove the enrichment broth from the incubator and gently agitate the contents.
3. One LS tube is required for each sample and the Negative Control (NC) sample.
   3.1 LS tube strips can be cut to desired LS tube number. Select the number of individual LS tubes or 8-tube strips needed. Place the LS tubes in an empty rack.
   3.2 To avoid cross-contamination, decap one LS tubes strip at a time and use a new pipette tip for each transfer step.
4. Transfer enriched sample to LS tubes as described below:

Transfer each enriched sample into individual LS tube **first**. Transfer the NC **last**.

   4.1 Use the 3M™ Molecular Detection Cap/Decap Tool-Lysis to decap one LS tube strip - one strip at a time. Set the tool with cap attached aside on a clean surface.
   4.2 Transfer 20 µL of sample into a LS tube.

4.3 Repeat step 4.2 until each individual sample has been added to a corresponding LS tube in the strip

4.4 Use the 3M Molecular Detection Cap/Decap Tool-Lysis to re-cap the LS tube strip. Use the rounded side of the tool to apply pressure in a back and forth motion ensuring that the cap is tightly applied.

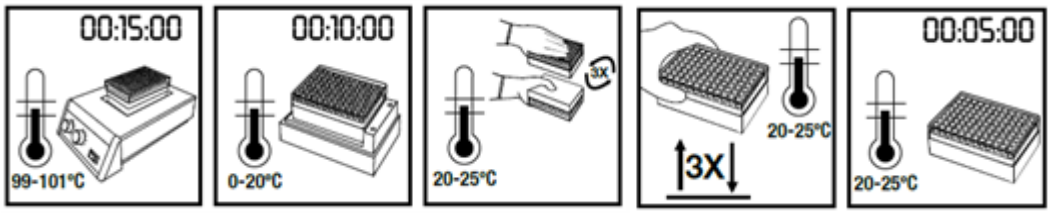


4.5 Repeat steps 4.1 to 4.4 as needed, for the number of samples to be tested

4.6 When all samples have been transferred, then transfer **<u>20 µL of NC</u> into a LS tube**. Use the 3M Molecular Detection Cap/Decap Tool-Lysis tool to re-cap the LS tube.

4.7 Cover the rack of LS tubes with the rack lid and firmly invert 3-5 times to mix. **Suspension has to flow freely inside the tube.**

5. Verify that the temperature of the 3M Molecular Detection Heat Block Insert is at 100 ±1°C. Place the rack of LS tubes in the 3M Molecular Detection Heat Block Insert and heat for 15 ±1 minutes [(b)]. Samples that have not been properly heat treated during the assay lysis step may be considered a potential biohazard and should NOT be inserted into the 3M Molecular Detection Instrument.
6. Remove the rack of LS tubes from the heating block and allow to cool in the 3M Molecular Detection Chill Block Insert for 10 ±1 minutes [(c)]. **Remove the rack lid during incubation on the 3M Molecular Detection Chill Block Insert**.
7. Remove the rack of LS tubes from the 3M Molecular Detection Chill Block Insert/ 3M Molecular Detection Chill Block Tray system. Replace the lid on the rack of LS tubes and firmly invert 3-5 times to mix. **Suspension has to flow freely inside the tube.**
8. Firmly tap the lysis tubes rack on the laboratory bench 3-5 times.
9. Place the rack on the laboratory bench. Let it sit undisturbed for at least 5 minutes to allow the resin to settle. **<u>Do not mix or disturb the resin at the bottom of the tube</u>**.



(a) Alternatives to equilibrate the LS tubes to room temperature are to incubate the LS tubes in a 37 ±1°C incubator for 1 hour or at room temperature overnight (16-18 hours).

(b) An alternative to using dry heat for the lysis step is to use a water bath at 100 ±1°C. Ensure that sufficient water is used to cover up to the liquid level in the LS tubes. Place the rack of LS tubes in the water bath at 100 ±1°C and heat for 15 ±1 minutes.

(c) The LS solution may freeze when processing less than 48 LS tubes. Freezing of the LS solution will not affect your test. If freezing is observed, allow the LS tubes to thaw for 5 minutes before mixing.

J. AMPLIFICATION

1. One Reagent tube is required for each sample and the NC.

1.1 Reagent tubes strips can be cut to desired tube number. Select the number of individual Reagent tubes or 8-tube strips needed.

1.2 Place Reagent tubes in an empty rack.

1.3 Avoid disturbing the reagent pellets from the bottom of the tubes.

2. Select 1 Reagent Control (RC) tube and place in rack.
3. To avoid cross-contamination, decap one Reagent tubes strip at a time and use a new pipette tip for each transfer step.
4. Transfer lysate to Reagent tubes and RC tube as described below:

Transfer each sample lysate into individual Reagent tubes **first** followed by the NC. Hydrate the RC tube **last**.
WARNING: Care must be taken when pipetting LS, as carry-over of the resin may interfere with amplification.

4.1 Use the 3M™ Molecular Detection Cap/Decap Tool-Reagent to decap the Reagent tubes –one Reagent tubes strip at a time. Discard cap.

4.2 Transfer 20 µL of Sample lysate from the upper portion of the fluid in the LS tube into corresponding Reagent tube. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down 5 times.

4.3 Repeat step 4.2 until individual Sample lysate has been added to a corresponding Reagent tube in the strip.

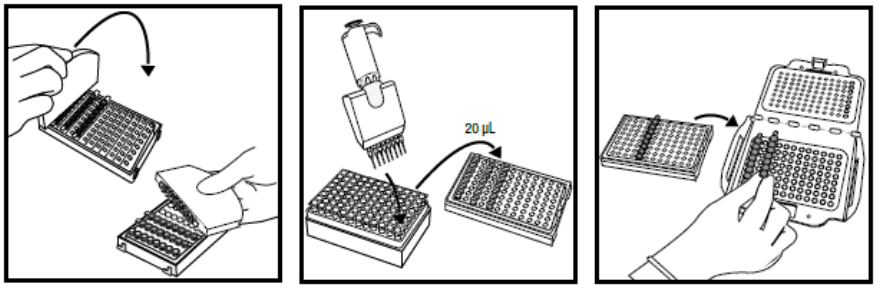
4.4 Cover the Reagent tubes with the provided extra cap and use the rounded side of the 3M Molecular Detection Cap/Decap Tool-Reagent to apply pressure in a back and forth motion ensuring that the cap is tightly applied.

4.5 Repeat steps 4.1 to 4.4 as needed, for the number of samples to be tested.

4.6 When all sample lysates have been transferred, repeat 4.1 to 4.4 to transfer 20 µL of NC lysate into a Reagent tube.

4.7 Transfer **20 µL of <u>NC lysate</u> into a RC tube**. Dispense at an angle to avoid disturbing the pellets. Mix by gently pipetting up and down 5 times.

5. Load capped tubes into a clean and decontaminated 3M Molecular Detection Speed Loader Tray. Close and latch the 3M Molecular Detection Speed Loader Tray lid.



6. Review and confirm the configured run in the 3M Molecular Detection Software.
7. Click the Start button in the software and select instrument for use. The selected instrument's lid automatically opens.
8. Place the 3M Molecular Detection Speed Loader Tray into the 3M Molecular Detection Instrument and close the lid to start the assay. Results are provided within 75 minutes, although positives may be detected sooner.
9. After the assay is complete, remove the 3M Molecular Detection Speed Loader Tray from the 3M Molecular Detection Instrument and dispose of the tubes by soaking in a 1-5% (v:v in water) household bleach solution for 1 hour and away from the assay preparation area.

NOTICE: To minimize the risk of false positives due to cross-contamination, never open reagent tubes containing amplified DNA. This includes Reagent Control, Reagent and Matrix Control tubes. Always dispose of sealed reagent tubes by soaking in a 1-5% (v:v in water) household bleach solution for 1 hour and away from the assay preparation area.

K. RESULTS AND INTERPRETATION

An algorithm interprets the light output curve resulting from the detection of the nucleic acid amplification. Results are analyzed automatically by the software and are color-coded based on the result. A Positive or Negative result is determined by analysis of a number of unique curve parameters. Presumptive positive results are reported in real-time while Negative and Inspect results will be displayed after the run is completed. Presumptive positive results should be confirmed using your preferred method or as specified by theFDA/BAM, theUSDA/FSIS-MLG, the AOAC OMA 993.12 or the ISO 11290 methodsstarting from the 3M Primary enrichment, followed by secondary enrichment or direct plating and confirmation of isolates using appropriate biochemical and serological methods.

NOTE: Even a negative sample will not give a zero reading as the system and 3M Molecular Assay *Listeria monocytogenes*amplification reagents have a "background" relative light unit (RLU).

In the rare event of any unusual light output, the algorithm labels this as "Inspect." 3M recommends the user to repeat the assay for any Inspect samples. If the result continues to be Inspect, proceed to confirmation test using your preferred method or as specified by local regulations.

Results of Collaborative Study

For this collaborative study, the 3M Molecular Detection Assay (MDA) *Listeria monocytogenes* method was compared to the to the AOAC 993.12 reference method for full fat cottage cheese and to the USDA/FSIS-MLG 8.09 for deli turkey. A total of 16 laboratories throughout the United States and Canada participated in this study, with 13 laboratories submitting data for the full fat cottage cheese and 11 laboratories submitting data for deli turkey. Each laboratory analyzed 36 test portions for each method: 12 inoculated with a high level of *Listeria monocytogenes*, 12 inoculated with a low level of *Listeria monocytogenes*, and 12 un-inoculated controls.The 3M™ MDA *Listeria monocytogenes* method produced 394 presumptive positive results with 392 confirming positive. There were 403 confirmed positives by the reference method.

A background screen of the matrices indicated an absence of indigenous *Listeria*species, including *Listeria monocytogenes*. For each matrix, the level of *Listeria monocytogenes* was determined by MPN determination on the day of initiation of analysis by the coordinating laboratory. The results of the heat stress injury for the deli turkey inoculum are presented in Table 2. The individual laboratory and sample results are presented in Tables 3 and 4. Tables2014.1A-1Bsummarizes the inter-laboratory results for all foods tested, including POD statistical analysis [10]. As per criteria outlined in Appendix J of the AOAC Validation Guidelines, fractional positive results were obtained.Detailed results for each laboratory are presented in Tables 2014.2A-2B and Figures 1A-B and 2A-Bof the Supplemenatary materials. The results for each collaborating laboratory's 3M Petrifilm Aerobic Count Plate (OMA 990.12) are presented in Table 2014.3 of the Supplemenatary materials.

Full Fat Cottage Cheese Results (25 g Test Portions)

Full fat cottage cheese test portions were inoculated at a low and high level and were analyzed (Table 2) for the detection of *Listeriamonocytogenes*. Un-inoculated controls were included in each analysis. Fifteen Laboratories participated in the evaluation of the full fat cottage cheese. Laboratories 4 and 5 did not submit results to the coordinating laboratory. Laboratories 6 and 13 reported deviations in the protocol (laboratory6 incorrectly incubated their MDA test portions at 30°C instead for 48 hours instead of the required 37°C for 24 hours; laboratory 13 confirmed all colony growthregardless of supplementary test results (Gram stain, catalase reaction) indicating that the organism would not be classified as *Listeria* (Gram negative or Gram positive with spores, catalase negative))and results from these laboratories were excluded from the statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.80 CFU/test portion (0.63, 1.00) for the low level and 4.83 CFU/test portion (3.30, 7.70) for the high level.

For the high level, 129 out of 132 test portions (POD_{CP} of 0.98) were reported as presumptive positive by the 3M MDA *Listeriamonocytogenes*method with all 132 test portions (POD_{CC} of 1.00) confirming positive. Based on the valid data submitted from each of the collaborating laboratories, 3 false negative results were obtained resulting in 129 confirmed positives (POD_C of 0.98). [Using the POD statistical analysis, only presumptive positive samples that confirmed positive are used to calculate POD_C.] For the low level, 66 out of 132 test portions ($POD_{CP}$of 0.50) were reported as presumptive positive by the 3M MDA *Listeria monocytogenes*method with 64 test portions (POD_{CC}of0.48) confirming positive. Based on the valid data submitted from each of the collaborating laboratories, 3 false positive results and 1 false negative result were obtained resulting in 63 confirmed positives (POD_C of 0.47).For the un-inoculated controls, 0 out of 132 samples produced a presumptive positive result by the 3M MDA *Listeria monocytogenes*method with all test portions confirming negative. For test portions analyzed by the AOAC 993.12 Method, 132 out of 132 high leveltest portions (POD_R of 1.00) and 73 out of 132 low leveltest portions (POD_R of 0.55) confirmed positive. For the un-inoculated controls, 0 out of 132 test portions (POD_R of 0.00) confirmed positive.

For the low level, a $dLPOD_C$ value of -0.08 with 95% confidence intervals of (-0.20, 0.05) were obtained between the 3M MDA *Listeriamonocytogenes*method and the AOAC OMA 993.12 method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.02 with 95% confidence intervals of (-0.11, 0.14) were obtained between presumptive and confirmed 3M MDA *Listeriamonocytogenes*results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high level, a $dLPOD_C$ value of -0.02 with 95% confidence intervals of (-0.06, 0.01) were obtained between the 3M MDA *Listeria monocytogenes*method and the AOAC OMA 993.12 method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of -0.02 with 95% confidence intervals of (-0.06, 0.01) were obtained between presumptive and confirmed 3M MDA *Listeriamonocytogenes*results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table 2014.2A and Figures 1A-1B of the Supplemenatary materials.

Deli Turkey Results (125 g Test Portions)

Deli turkey test portions were inoculated at a low and high level and were analyzed (Table 2) for the detection of *Listeria monocytogenes.*Fourteen laboratories participated in the analysis of the deli turkey. Un-inoculated controls were included in each analysis. Laboratory5did not submit results to the coordinating laboratory. Laboratory 8 reported a deviation in the protocol (Enrichment of MDA test portions using UVM broth) and laboratory 10 reported the occurrence of cross contamination of their samples (8 confirmed positive uninoculated reference method samples and 7 confirmed positive uninoculatedMDA method samples). Results from these laboratories were excluded from the statistical analysis. The MPN levels obtained for this test portion, with 95% confidence intervals, were 0.66 CFU/test portion (0.51, 0.83) for the low level and 5.08 CFU/test portion (3.39, 7.63) for the high level. For the high level, 132 out of 132 test portions (POD_{CP}of1.00) were reported as presumptive positive by the 3M MDA *Listeria monocytogenes* method with all 132 test portions (POD_{CC} of 1.00) confirming positive.Based on the valid data submitted from each of the collaborating laboratories, no false negative or false positive results were obtained resulting in 132 confirmed positives (POD_C of 1.00). For the low level, 66 out of 132 test portions (POD_{CP} of 0.50) were reported as presumptive positive by the 3M MDA *Listeria monocytogenes* method with 67 test portions (POD_{CC} of 0.51) confirming positive. Based on the valid data submitted from each of the collaborating laboratories, 3 false negative results and 2 false positive results were obtained resulting in 64 confirmed positives (POD_C of 0.48).For the un-inoculated controls, 2 out of 132 samples (POD_{CP} of 0.02) produced a presumptive positive result by the 3M MDA *Listeria monocytogenes* method with one test portion(POD_{CC} of 0.01) confirming positive. (Each discrepant result was produced by a different laboratory. Based on the valid data submitted from each of the collaborating laboratories, 2 false negative results and 1 false positive results were obtained resulting in 64 confirmed positives (POD_C of 0.00). Laboratories 4 and 6 produced 1 false positive result and laboratory 16 produced 1 false negative result). For test portions analyzed by the USDA/FSIS-MLG Method, 132 out of 132 high inoculum (POD_R of 1.00) and 66 out of 132 low inoculum test portions (POD_R of 0.50) confirmed positive. For the un-inoculated controls, 0 out of 132 test portions (POD_R of 0.00) confirmed positive.

For the low level, a $dLPOD_C$ value of -0.02 with 95% confidence intervals of (-0.14, 0.11) were obtained between the 3M MDA *Listeria monocytogenes* method and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of -0.01 with 95% confidence intervals of (-0.13, 0.12) were obtained between presumptive and confirmed 3M MDA *Listeria monocytogenes* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results using either confirmation process.

For the high level, a $dLPOD_C$ value of 0.00 with 95% confidence intervals of (-0.03, 0.03) were obtained between the 3M MDA *Listeria monocytogenes* method and the USDA/FSIS-MLG method. The confidence intervals obtained for $dLPOD_C$ indicated no significant difference between the two methods. A $dLPOD_{CP}$ value of 0.00 with 95% confidence intervals of (-0.03, 0.03) were obtained between presumptive and confirmed 3M MDA *Listeria monocytogenes* results. The confidence intervals obtained for $dLPOD_{CP}$ indicated no significant difference between the presumptive and confirmed results. Detailed results of the POD statistical analysis are presented in Table 2014.2B and Figures 2A-2B of the Supplemenatary aterials.

Discussion

No negative feedback was provided by the collaborating laboratories in regards to the performance of the 3M MDA *Listeria monocytogenes*.Several laboratories reported difficulty in isolating and identifying *Listeria* colonies on Oxford agar (OXA) from samples enriched in the DF broth base (without FAC) when compared to samples enriched in the AOAC OMA 991.12 selective enrichment broth. This may be related to differences in formulation between the two enrichments. The AOAC OMA 993.12 enrichment broth is designed to reduce the background flora on OXA and is more selective than DF broth base (without FAC). In some instances, this level of selectivity may cause stress on *Listeria* cells thus requiring a longer enrichment time to reach a detectable level.

Based on the data submitted, 2 laboratories were removed from statistical consideration for both the full fat cottage cheese and the deli turkey. For the cottage cheese, laboratory 6 did not follow the approved incubation time andtemperature conditions for the candidate method (samples were incubated for 48 hours at 30°C and the validated enrichment time and temperature are 24 to 28 hours at 37°C.) and laboratory 12 confirmed growth from all plates, regardless of supplementary tests that would have precluded confirmation via API *Listeria*.Due to this fact, all samples confirmed via API *Listeria* produced a *Listeria* species result even if Gram reaction (Gram negative), motility reaction (negative), catalase reaction (negative)andoxidase reaction (positive) would indicate the orgnaisms is not of the genus*Listeria*. For the deli turkey, laboratory 8 incorrectly enriched half of their candidate method samples using the reference method enrichment broth (UVM) instead of DF broth. Laboratory 10 reported more confirmed positive results in their uninoculated control samples (for both the candidate and reference method) then for their low level contamination level indicating a substantial level of laboratory cross-contamination. Based on these results, these laboratories were removed from statistical analysis. No laboratories were removed from statistical analysis based on discrepancies between presumptive and confirmed results.

During the collaborative study evaluation 7 false positive (3 for full fat cottage cheese and 4 for deli turkey) and 8 false negative (4 for full fat cottage cheese and 4 for deli turkey) were obtained out of 792 total test portions analyzed by the 3M MDA *Listeria monocytogenes*. The candidate methodcorrectly identified whether a test portion was positive or negative more than 98.1% of the time (false positive rate of 0.9% and false negative rate of 1.0%). Several of the false positive discrepant results were obtained from uninoculated control test portions and several of the false negative discrepant results were obtained from high level inoculated test portions which may indicate that they are the result of laboratory error and not performance of the assay. No evidence of physical cause or suspicion of cause was noted and it was determined that they would be included in the statistical analysis.

For each matrix, the collaborative study indicated that no statistically significant difference between the candidate method and the reference methods or the presumptive and confirmed results of the candidate method was obtained when using the POD statistical model.

Recommendations

It is recommended that the 3M Molecular Detection Assay *Listeria monocytogenes*method be adopted as Official First Action status for the detection of *Listeria monocytogenes* in selected foods includingbeef hot dogs (25 g & 125 g), deli turkey (25 g & 125 g), cold smoked salmon (25 g), full fat cottage cheese (25 g), chocolate milk (25 g), and two environmental surfaces,

sealed concrete (sponge in 100 mL and sponge in 225 mL) and stainless steel (sponge in 225 mL)

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Table 1: Participation of each Collaborating Laboratory[a]

Lab	Full Fat Cottage Cheese[a]	Deli Turkey[a]
1	Y	Y
2	Y	Y
3	Y	Y
4	Y[c]	Y
5	Y[c]	Y[c]
6	Y[b]	Y
7	Y	Y
8	Y	Y[b]
9	Y	N
10	Y	Y[b]
11	Y	Y
12	Y	N
13	Y[b]	Y
14	Y	Y
15	Y	Y
16	N	Y

[a] Y= Collaborator analyzed the food type; N= collaborator did not analyze the food type
[b] Results were not used in statistical analysis due to laboratory error
[c]Results were not submitted to the coordinating laboratory

Table 2: Heat-Stress Injury Results

Matrix	Test Organism[a]	CFU/MOX (Selective Agar)	CFU/TSA/ye (Non-Selective Agar)	Degree Injury[b]
Deli Turkey	*Listeria monocytogenes* ATCC 13932	7.7×10^8	3.2×10^9	76.0%

[a] ATCC- American Type Culture Collection
[b] Cultures were heat stressed for 10 minutes at 50°C in a circulating water bath.

Table 3. Individual Collaborator Results for Full Fat Cottage Cheese (4% Milk Fat)

	High-Level Test Portions												Low Level Test Portions												Un-inoculated Test Portions											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
3M MDA *Listeria monocytogenes*																																				
Lab																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	-[c]	+	+	-	-	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+	+	+	-[c]	-	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
4[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
6[b]	+	+	+	+	+	+	+	+	+	+	+	+	+[d]	+[d]	+[d]	+[d]	+	+	+	+	+	+[d]	+	+	+[d]	+[d]	+[d]	+[d]	+	+[d]	+[d]	+[d]	+[d]	+[d]	+[d]	+[d]
7	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	+[d]	-	-	-	-	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-[c]
12	+	+	+[d]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13[b]	+	+	+	+	+	+	+	+[d]	+[d]	+	+	+	+	-	+	+[d]	+[d]	+[d]	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
15	+	+	+	+	+	+[d]	+[d]	+	+	+	+	+	+	-	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
16	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
AOAC OMA 993.12																																				
Lab																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
5[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
6[b]	+	+	+	+	+	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
8	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
9	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
10	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
12	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
13[b]	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
15	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
16	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a?

[a] += *Listeriamonocytogenes* were detected in samples; -=*Listeriamonocytogenes* were not detected in sample; n/a- lab did not participate in this matrix or results were not received

[b] Results were not used in statistical analysis due to laboratory error; [c] Sample was presumptive positive on 3M MDA *Listeria monocytogenes* but confirmed negative indicating a false positive result;[d]Sample was presumptive negative on 3M MDA *Listeria monocytogenes* but confirmed positive indicating a false negative result

Table 4. Individual Collaborator Results for Deli Turkey

	High-Level Test Portions												Low Level Test Portions												Un-inoculated Test Portions											
	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12
3M MDA *Listeria monocytogenes*																																				
Lab																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+[d]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+[d]	-	-	+	-	-	-	-	-	-	-	-	-[c]	-	-	-	-
5[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
6	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-[c]	-	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-[c]	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	n/a	-	n/a	+	+	+	-	-	-	-	-	-	-	-[c]	+	n/a	n/a	n/a	-	+
9	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
10[b]	+	+	+	+	+	+[d]	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+[d]	+	+	+	-	-	-	+[d]	+	-	-
11	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	-	-	-	+[d]	-	-	-	-	-	-	-	-	-	-	-	-
12[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	-[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
16	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-	+[d]	-	-	-	-	-	-	-
USDA/FSIS – MLG 8.09																																				
1	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
3	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-
5[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
6	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-		+	+	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
8[b]	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
9	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
10[b]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	-	+	+	-	+	-
11	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
12[b]	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
13	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
15	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
16	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] += *Listeriamonocytogenes* were detected in samples; -=*Listeria monocytogenes* were not detected in sample; n/a- lab did not participate in this matrix or results were not received

[b] Results were not used in statistical analysis due to laboratory error; [c] Sample was presumptive positive on 3M MDA *Listeria monocytogenes* but confirmed negative indicating a false positive result;[d]Sample was presumptive negative on 3M MDA *Listeria monocytogenes*but confirmed positive indicating a false negative result

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Table 2014.1A: POD Summary of Full Fat Cottage CheeseResults for the 3M Molecular Detection Assay *Listeria monocytogenes*

Method[a]	**3M™ MDA™ *Listeria monocytogenes***		
Inoculation Level	**Uninoculated**	**Low**	**High**
Candidate Presumptive Positive/ Total # of Samples Analyzed	0/132	66/132	129/132
Candidate Presumptive POD (CP)	0.00 (0.00, 0.03)	0.50 (0.41, 0.59)	0.98 (0.94, 0.99)
s_r[b]	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.15 (0.13, 0.17)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.17)	0.03 (0.00, 0.08)
s_R[d]	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.15 (0.13, 0.18)
P Value[e]	1.0000	0.9123	0.9499
Candidate Confirmed Positive/ Total # of Samples Analyzed	0/132	64/132	132/132
Candidate Confirmed POD (CC)	0.00 (0.00, 0.03)	0.48 (0.40, 0.57)	1.00 (0.97, 1.00)
s_r[b]	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.15)	0.00 (0.00, 0.16)
s_R[d]	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P Value[e]	1.0000	0.8762	1.0000
Candidate Confirmed Positive/ Total # of Samples Analyzed	0/132	63/132	129/132
Candidate Confirmed POD (C)	0.00 (0.00, 0.03)	0.48 (0.39, 0.57)	0.98 (0.94, 0.99)
s_r[b]	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.15 (0.13, 0.17)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.14)	0.03 (0.00, 0.08)
s_R[d]	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.15 (0.13, 0.18)
P Value[e]	1.0000	0.9080	0.9499
Positive Reference Samples/ Total # of Samples Analyzed	0/132	73/132	132/132
Reference POD	0.00 (0.00, 0.03)	0.55 (0.47, 0.64)	1.00 (0.97, 1.00)
s_r[b]	0.00 (0.00, 0.16)	0.50 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.18)	0.00 (0.00, 0.16)
s_R[d]	0.00 (0.00, 0.23)	0.50 (0.45, 0.52)	0.00 (0.00, 0.23)
P Value[e]	1.0000	0.6678	1.0000
dLPOD (Candidate vs. Reference)[f]	0.00 (-0.03, 0.03)	-0.08 (-0.20, 0.05)	-0.02 (-0.06, 0.01)
dLPOD (Candidate Presumptive vs. Candidate Confirmed)[f]	0.00 (-0.03, 0.03)	0.02(-0.11, 0.14)	-0.02 (-0.06, 0.01)

[a] Results include 95% Confidence Intervals, [r] Repeatability Standard Deviation, [c] Among-Laboratory Standard Deviation, [d] Reproducibility Standard Deviation

[e] P Value = Homogeneity test of laboratory PODs; [f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods

Table 2014.1B: POD Summary of Deli Turkey Results for the 3M Molecular Detection Assay *Listeria monocytogenes*

Method[a]	3M™ MDA™ *Listeria monocytogenes*		
Inoculation Level	**Uninoculated**	**Low**	**High**
Candidate Presumptive Positive/ Total # of Samples Analyzed	2/132	66/132	132/132
Candidate Presumptive POD (CP)	0.02 (0.01, 0.05)	0.50 (0.41, 0.59)	1.00 (0.97, 1.00)
s_r[b]	0.12 (0.11, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.05)	0.00 (0.00, 0.17)	0.00 (0.00, 0.16)
s_R[d]	0.12 (0.11, 0.14)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P Value[e]	0.5190	0.7565	1.0000
Candidate Confirmed Positive/ Total # of Samples Analyzed	1/132	67/132	132/132
Candidate Confirmed POD (CC)	0.01 (0.00, 0.04)	0.51 (0.42, 0.60)	1.00 (0.97, 1.00)
s_r[b]	0.09 (0.08, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.04)	0.00 (0.00, 0.17)	0.00 (0.00, 0.16)
s_R[d]	0.09 (0.08, 0.10)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P Value[e]	0.4338	0.7891	1.0000
Candidate Confirmed Positive/ Total # of Samples Analyzed	0/132	64/132	132/132
Candidate Confirmed POD (C)	0.00 (0.00, 0.03)	0.48 (0.40, 0.57)	1.00 (0.97, 1.00)
s_r[b]	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)	0.00 (0.00, 0.16)
s_R[d]	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P Value[e]	1.0000	0.8249	1.0000
Positive Reference Samples/ Total # of Samples Analyzed	0/132	66/132	132/132
Reference POD	0.00 (0.00, 0.03)	0.50 (0.41, 0.59)	1.00 (0.97, 1.00)
s_r[b]	0.00 (0.00, 0.16)	0.51 (0.45, 0.52)	0.00 (0.00, 0.16)
s_L[c]	0.00 (0.00, 0.16)	0.00 (0.00, 0.17)	0.00 (0.00, 0.16)
s_R[d]	0.00 (0.00, 0.23)	0.51 (0.46, 0.52)	0.00 (0.00, 0.23)
P Value[e]	1.0000	0.7565	1.0000
dLPOD (Candidate vs. Reference)[f]	0.00 (-0.03, 0.03)	-0.02 (-0.14, 0.11)	0.00 (-0.03, 0.03)
dLPOD (Candidate Presumptive vs. Candidate Confirmed)[f]	0.01 (-0.03, 0.05)	-0.01 (-0.13, 0.12)	0.00 (-0.03, 0.03)

[a] Results include 95% Confidence Intervals, [r] Repeatability Standard Deviation, [c] Among-Laboratory Standard Deviation, [d] Reproducibility Standard Deviation
[e] P Value = Homogeneity test of laboratory PODs; [f] A confidence interval for dLPOD that does not contain the value 0 indicates a statistical significant difference between the two methods

Supplemenatary Materials

Table 2014.2A: Comparative Results for the Detection of *Listeriamonocytogenes*in Full Fat Cottage Cheese (4% Milk Fat) by the 3M™ MDA *Listeriamonocytogenes* Method vs. AOAC OMA 993.12 in a Collaborative Study (Uninoculated Control)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD (CP)	N	X	POD (CC)	N	X	POD (C)	N	X	POD (R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		2	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		3	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		4[a]	NA[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[a]	12	1	0.08	12	12	1.00	12	1	0.08	12	0	0.00	0.08	1.00
		7	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		8	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		9	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		10	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		11	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		12	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		13[a]	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		14	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		15	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
Estimate		All	132	0	0.00	132	0	0.00	132	0	0.00	132	0	0.00	0.00	0.00
LCL					0.00			0.00			0.00			0.00	-0.03	-0.03
UCL					0.03			0.03			0.03			0.03	0.03	0.03
s_r[b]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.16			0.16			0.16			0.16		
s_L[c]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.16			0.16			0.16			0.16		
s_R[d]					0.00			0.00			0.00			0.00		
UCL					0.00			0.00			0.00			0.00		
LCL					0.23			0.23			0.23			0.23		
P_T[e]					1.0000			1.0000			1.0000			1.0000		

[a] Results were not used in statistical analysis due to deviation from testing protocol.

[b] Repeatability Standard Deviation, [c] Among-Laboratory Standard Deviation, [d] Reproducibility Standard Deviation, [e] P_T Value =Homogeneity test of laboratory PODs,[f]NA- laboratory results were not received

LCL – Lower Confidence Limit; UCL – Upper Confidence Limit

Table 2014.2A (cont'd.): Comparative Results for the Detection of *Listeriamonocytogenes*in Full Fat Cottage Cheese (4% Milk Fat) by the 3M™ MDA *Listeriamonocytogenes*Method vs. AOAC OMA 993.12 in a Collaborative Study (Low Inoculum Level)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD(CP)	N	X	POD(CC)	N	X	POD(C)	N	X	POD(R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	6	0.50	12	4	0.33	12	4	0.33	12	6	0.50	-0.17	0.08
		2	12	7	0.58	12	7	0.58	12	7	0.58	12	7	0.58	0.00	0.00
		3	12	8	0.67	12	7	0.58	12	7	0.58	12	7	0.58	0.09	0.00
		4[a]	NA[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[a]	12	7	0.58	12	12	1.00	12	7	0.58	12	2	0.17	0.41	-0.42
		7	12	4	0.33	12	4	0.33	12	4	0.33	12	10	0.83	-0.50	0.00
		8	12	6	0.50	12	7	0.58	12	6	0.50	12	7	0.58	-0.08	-0.08
		9	12	4	0.33	12	4	0.33	12	4	0.33	12	8	0.67	0.34	0.00
		10	12	6	0.50	12	6	0.60	12	6	0.60	12	7	0.58	0.08	0.00
		11	12	6	0.50	12	6	0.60	12	6	0.60	12	5	0.42	0.08	0.00
		12	12	6	0.50	12	6	0.50	12	6	0.50	12	5	0.42	0.08	0.00
		13[a]	12	4	0.33	12	7	0.58	12	4	0.33	12	7	0.58	-0.25	-0.25
		14	12	6	0.50	12	6	0.50	12	6	0.50	12	6	0.50	0.00	0.00
		15	12	7	0.58	12	7	0.58	12	7	0.58	12	5	0.42	0.16	0.00
Estimate	0.80	All	132	66	0.50	132	64	0.48	132	63	0.48	132	73	0.55	-0.08	0.02
LCL	0.63				0.41			0.40			0.39			0.47	-0.20	-0.11
UCL	1.00				0.59			0.57			0.57			0.64	0.05	0.14
s_r[b]					0.51			0.51			0.51			0.50		
LCL					0.45			0.45			0.45			0.45		
UCL					0.52			0.52			0.52			0.52		
s_L[c]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.17			0.15			0.14			0.18		
s_R[d]					0.51			0.51			0.51			0.50		
UCL					0.46			0.46			0.46			0.45		
LCL					0.52			0.52			0.52			0.52		
P_T[e]					0.9123			0.8762			0.9080			0.6678		

[a] Results were not used in statistical analysis due to deviation from testing protocol
[b] Repeatability Standard Deviation, [c] Among-Laboratory Standard Deviation, [d] Reproducibility Standard Deviation, [e] P_T Value =Homogeneity test of laboratory PODs,[f]NA- laboratory results were not received
LCL – Lower Confidence Limit; UCL – Upper Confidence Limit

Table 2014.2A (cont'd): Comparative Results for the Detection of *Listeria monocytogenes* in Full Fat Cottage Cheese (4% Milk Fat) by the 3M™ MDA *Listeriamonocytogenes*Method vs. AOAC OMA 993.12 in a Collaborative Study (High Inoculum Level)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD(CP)	N	X	POD(CC)	N	X	POD(C)	N	X	POD(R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		2	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		3	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		4[a]	NA[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		5[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6[a]	12	12	1.00	12	12	1.00	12	12	1.00	12	7	0.58	0.42	0.00
		7	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		8	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		9	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		10	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		11	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		12	12	11	0.92	12	12	1.00	12	11	0.92	12	12	1.00	-0.08	-0.08
		13[a]	12	10	0.83	12	12	1.00	12	10	0.83	12	12	1.00	-0.17	-0.17
		14	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		15	12	10	0.83	12	12	1.00	12	10	0.83	12	12	1.00	-0.17	-0.17
		16[a]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Estimate	4.83 3.30 7.07	All	132	129	0.98	132	132	1.00	132	129	0.98	132	132	1.00	-0.02	-0.02
LCL					0.94			0.97			0.94			0.97	-0.06	-0.06
UCL					0.99			1.00			0.99			1.00	0.01	0.01
s_r[b]					0.15			0.00			0.15			0.00		
LCL					0.13			0.00			0.13			0.00		
UCL					0.17			0.16			0.17			0.16		
s_L[c]					0.03			0.00			0.03			0.00		
LCL					0.00			0.00			0.00			0.00		

UCL	0.08	0.16	0.08	0.16	
s_R[d]	0.15	0.00	0.15	0.00	
UCL	0.13	0.00	0.13	0.00	
LCL	0.18	0.23	0.18	0.23	
P_T[e]	0.9499	1.0000	0.9499	1.0000	

[a] Results were not used in statistical analysis due to deviation from testing protocol.

[c] Repeatability Standard Deviation, [d] Among-Laboratory Standard Deviation, [e] Reproducibility Standard Deviation, [f] P_T Value =Homogeneity test of laboratory PODs,[f]NA- laboratory results were not received
LCL – Lower Confidence Limit; UCL – Upper Confidence Limit

Table 2014.2B: Comparative Results for the Detection of *Listeriamonocytogenes* in Deli Turkey by the 3M™ MDA *Listeria monocytogenes* Method vs. the USDA/FSIS-MLG 8.09 Method in a Collaborative Study (Uninoculated Control)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD(CP)	N	X	POD(CC)	N	X	POD(C)	N	X	POD(R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		2	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		3	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		4	12	1	0.08	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.08
		5[b]	NA[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6	12	1	0.08	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.08
		7	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		8[a]	9	3	0.33	9	2	0.22	9	2	0.22	12	0	0.00	0.22	0.11
		9[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		10[a]	12	5	0.42	12	7	0.58	12	5	0.42	12	8	0.67	-0.25	-0.16
		11	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		12[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		13	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		14	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		15	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		16	12	0	0.00	12	1	0.08	12	0	0.00	12	0	0.00	0.00	-0.08
Estimate		All	132	2	0.02	132	1	0.01	132	0	0.00	132	0	0.00	0.00	0.01
LCL					0.01			0.00			0.00			0.00	-0.03	-0.03
UCL					0.05			0.04			0.03			0.03	0.03	0.05

s_r[b]			0.12	0.09	0.00	0.00		
LCL			0.11	0.08	0.00	0.00		
UCL			0.16	0.16	0.16	0.16		
s_L[c]			0.00	0.00	0.00	0.00		
LCL			0.00	0.00	0.00	0.00		
UCL			0.05	0.04	0.16	0.16		
s_R[d]			0.12	0.09	0.00	0.00		
UCL			0.11	0.08	0.00	0.00		
LCL			0.14	0.10	0.23	0.23		
P_T[e]			0.5190	0.4338	1.0000	1.0000		

[a] Results were not used in statistical analysis due to deviation from testing protocol.

[c] Repeatability Standard Deviation, [d] Among-Laboratory Standard Deviation, [e] Reproducibility Standard Deviation, [f] P_T Value =Homogeneity test of laboratory PODs,[f]NA- laboratory results were not received

LCL – Lower Confidence Limit; UCL – Upper Confidence Limit

Table 2014.2B (cont'd): Comparative Results for the Detection of *Listeriamonocytogenes* in Deli Turkey by the 3M™ MDA *Listeria monocytogenes* Method vs. the USDA/FSIS-MLG 8.09 Method in a Collaborative Study (Low Level Inoculum)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD(CP)	N	X	POD(CC)	N	X	POD(C)	N	X	POD(R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	6	0.50	12	6	0.50	12	6	0.50	12	6	0.50	0.00	0.00
		2	12	5	0.42	12	6	0.50	12	5	0.42	12	6	0.50	-0.08	-0.08
		3	12	8	0.67	12	8	0.67	12	8	0.67	12	7	0.58	0.08	0.00
		4	12	8	0.67	12	9	0.75	12	8	0.67	12	6	0.50	0.17	-0.08
		5[b]	NA[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6	12	6	0.50	12	5	0.42	12	5	0.42	12	7	0.58	-0.16	0.08
		7	12	6	0.50	12	6	0.50	12	6	0.50	12	5	0.42	0.08	0.00
		8[a]	10	7	0.70	10	7	0.70	10	7	0.70	12	6	0.50	0.20	0.00
		9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		10[a]	12	6	0.50	12	6	0.50	12	6	0.50	12	11	0.92	-0.42	0.00
		11	12	4	0.33	12	5	0.42	12	4	0.33	12	6	0.50	-0.17	-0.09
		12[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		13	12	5	0.42	12	5	0.42	12	5	0.42	12	5	0.42	0.00	0.00
		14	12	8	0.67	12	7	0.58	12	7	0.58	12	7	0.58	0.00	0.09
	0.66	15	12	5	0.42	12	5	0.42	12	5	0.42	12	7	0.58	-0.16	0.00
	0.51	16	12	5	0.42	12	5	0.42	12	5	0.42	12	4	0.33	-0.08	-0.00
	0.83															
Estimate		All	132	66	0.50	132	67	0.51	132	64	0.48	132	66	0.50	-0.02	-0.01

LCL			0.41	0.42	0.40	0.41	-0.14	-0.13
UCL			0.59	0.60	0.57	0.59	0.11	0.12
s_r[b]			0.51	0.51	0.51	0.51		
LCL			0.45	0.45	0.45	0.45		
UCL			0.52	0.52	0.52	0.52		
s_L[c]			0.00	0.00	0.00	0.00		
LCL			0.00	0.00	0.00	0.00		
UCL			0.17	0.17	0.16	0.17		
s_R[d]			0.51	0.51	0.51	0.51		
UCL			0.46	0.46	0.46	0.46		
LCL			0.52	0.52	0.52	0.52		
P_T[e]			0.7565	0.7891	0.8249	0.7565		

[a] Results were not used in statistical analysis due to deviation from testing protocol.

[c] Repeatability Standard Deviation, [d] Among-Laboratory Standard Deviation, [e] Reproducibility Standard Deviation, [f] P_T Value =Homogeneity test of laboratory PODs,[f]NA- laboratory results were not received

LCL – Lower Confidence Limit; UCL – Upper Confidence Limit

Table 2014.2B (cont'd): Comparative Results for the Detection of *Listeriamonocytogenes* in Deli Turkey by the 3M™ MDA *Listeria monocytogenes* Method vs. the USDA/FSIS-MLG 8.09 Method in a Collaborative Study (High Level Inoculum)

Statistic	MPN/ Test Portion	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs. R	
			N	X	POD(CP)	N	X	POD(CC)	N	X	POD(C)	N	X	POD(R)	dLPOD (C,R)	dLPOD (CP,CC)
		1	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		2	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		3	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		4	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		5[b]	NA[f]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		6	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		7	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		8[a]	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		9[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		10[a]	12	11	0.92	12	12	1.00	12	11	0.92	12	12	1.00	-0.08	-0.08
		11	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
		12[b]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		13	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
	5.08	14	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
	3.39	15	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00
	7.63	16	12	12	1.00	12	12	1.00	12	12	1.00	12	12	1.00	0.00	0.00

Estimate		All	132	132	1.00	132	132	1.00	132	132	1.00	132	132	1.00	0.00	0.00
LCL					0.97			0.97			0.97			0.97	-0.03	-0.03
UCL					1.00			1.00			1.00			1.00	0.03	0.03
s_r[b]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.16			0.16			0.16			0.16		
s_L[c]					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.16			0.16			0.16			0.16		
s_R[d]					0.00			0.00			0.00			0.00		
UCL					0.00			0.00			0.00			0.00		
LCL					0.23			0.23			0.23			0.23		
P_T[e]					1.0000			1.0000			1.0000			1.0000		

[a] Results were not used in statistical analysis due to deviation from testing protocol.

[c] Repeatability Standard Deviation, [d] Among-Laboratory Standard Deviation, [e] Reproducibility Standard Deviation, [f] P_T Value =Homogeneity test of laboratory PODs,[f]NA- laboratory results were not received

LCL – Lower Confidence Limit; UCL – Upper Confidence Limit

Table 2014.2C: Results of Aerobic Plate Count for Collaborating Laboratories

Lab	Full Fat Cottage Cheese (CFU/g)	Deli Turkey (CFU/g)
1	1.0×10^1	3.8×10^4
2	7.2×10^3	1.5×10^3
3	1.0×10^1	<10
4	Not Reported	5.5×10^2
5	N/A[a]	N/A[a]
6	1.5×10^1	<10
7	<10	<10
8	3.0×10^2	1.5×10^3
9	1.2×10^2	N/A[a]
10	4.2×10^3	3.1×10^2

11	<10	<10
12	5.0×10^1	N/A[a]
13	8.5×10^1	<10
14	3.2×10^3	<10
15	1.2×10^2	<10
16	N/A[a]	2.0×10^1

[a]N/A – Laboratory did not participate in the analysis of matrix

Figure 1A: POD Values of Candidate Method vs. Reference Method

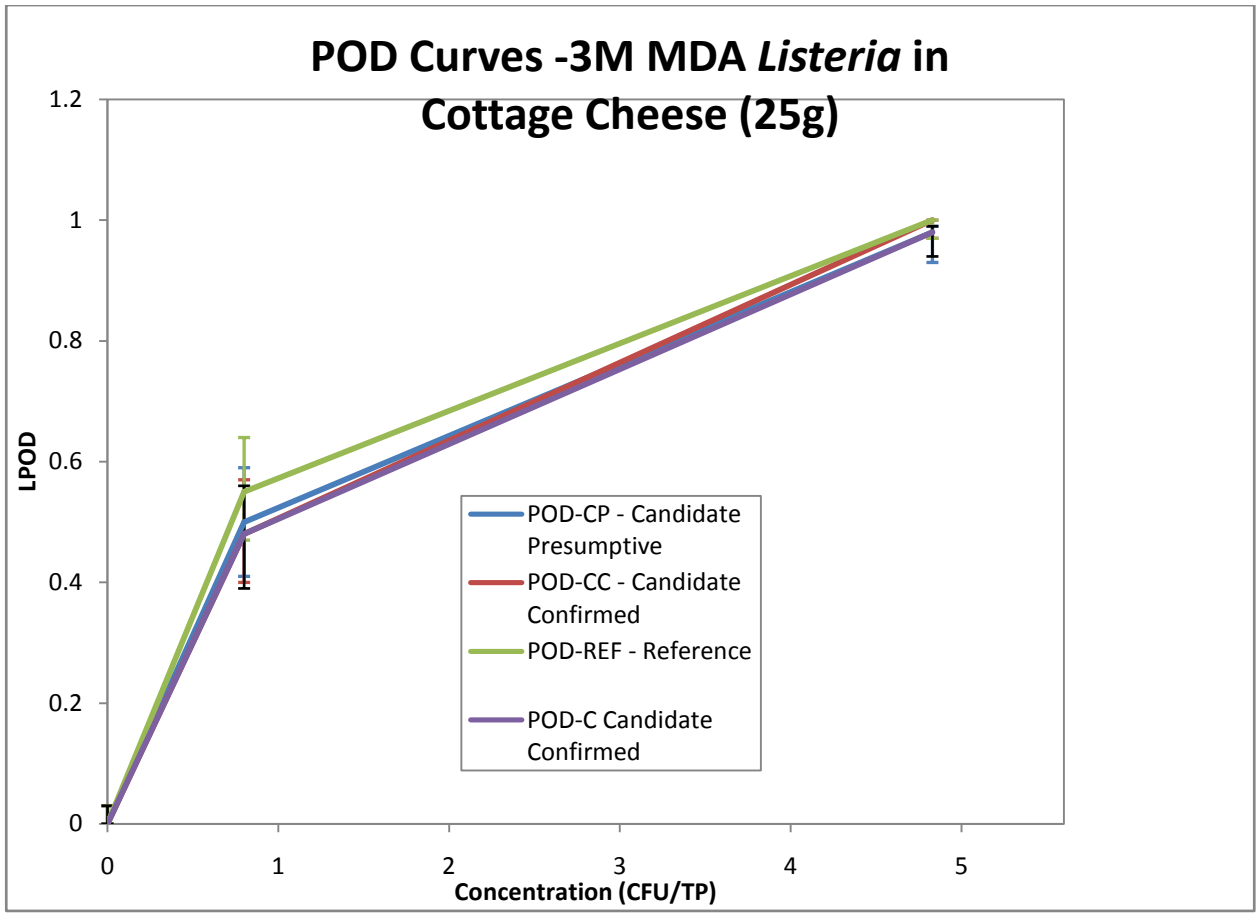


Figure 1B: dPOD Values of Candidate Method vs. Reference Method

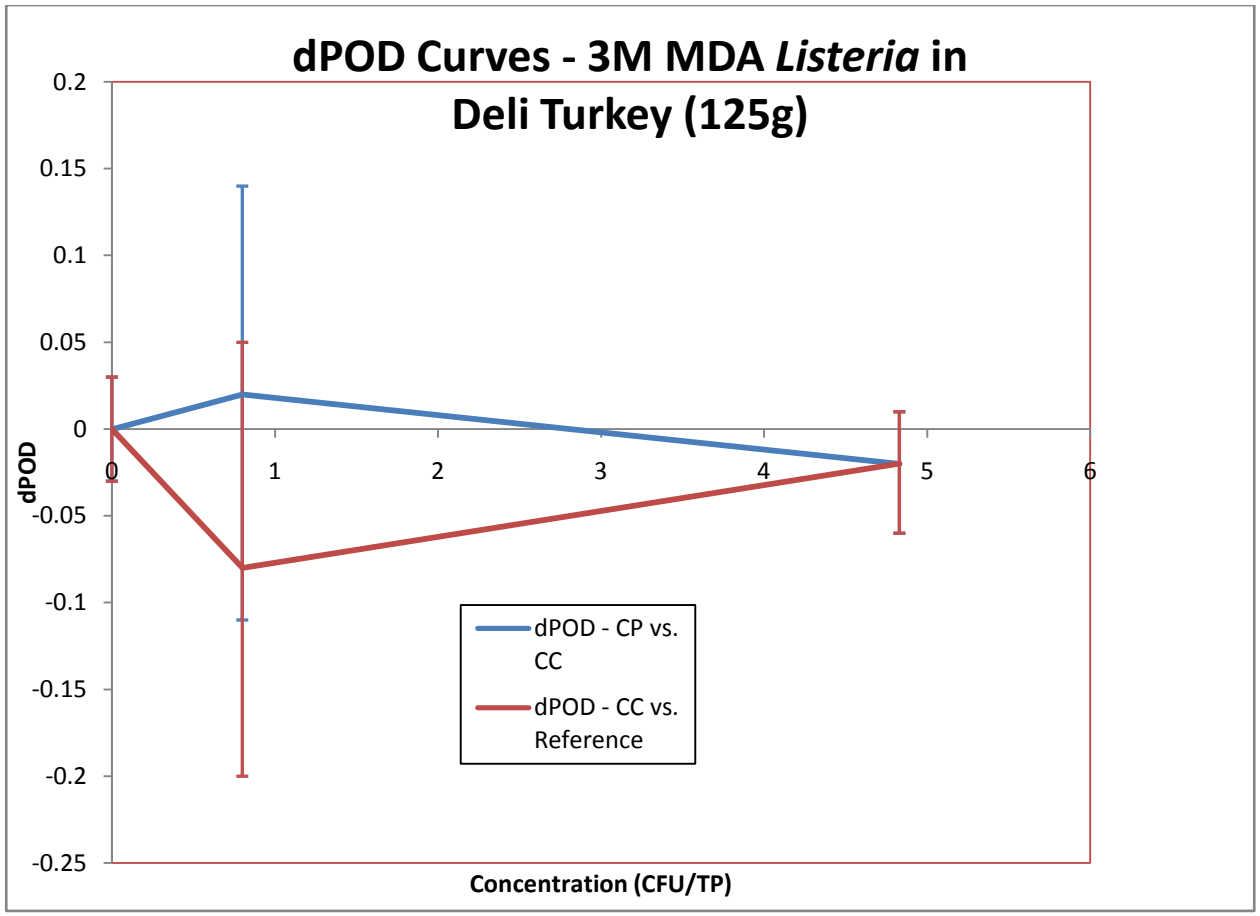


Figure 2A: POD Values of Candidate Method vs. Reference Method

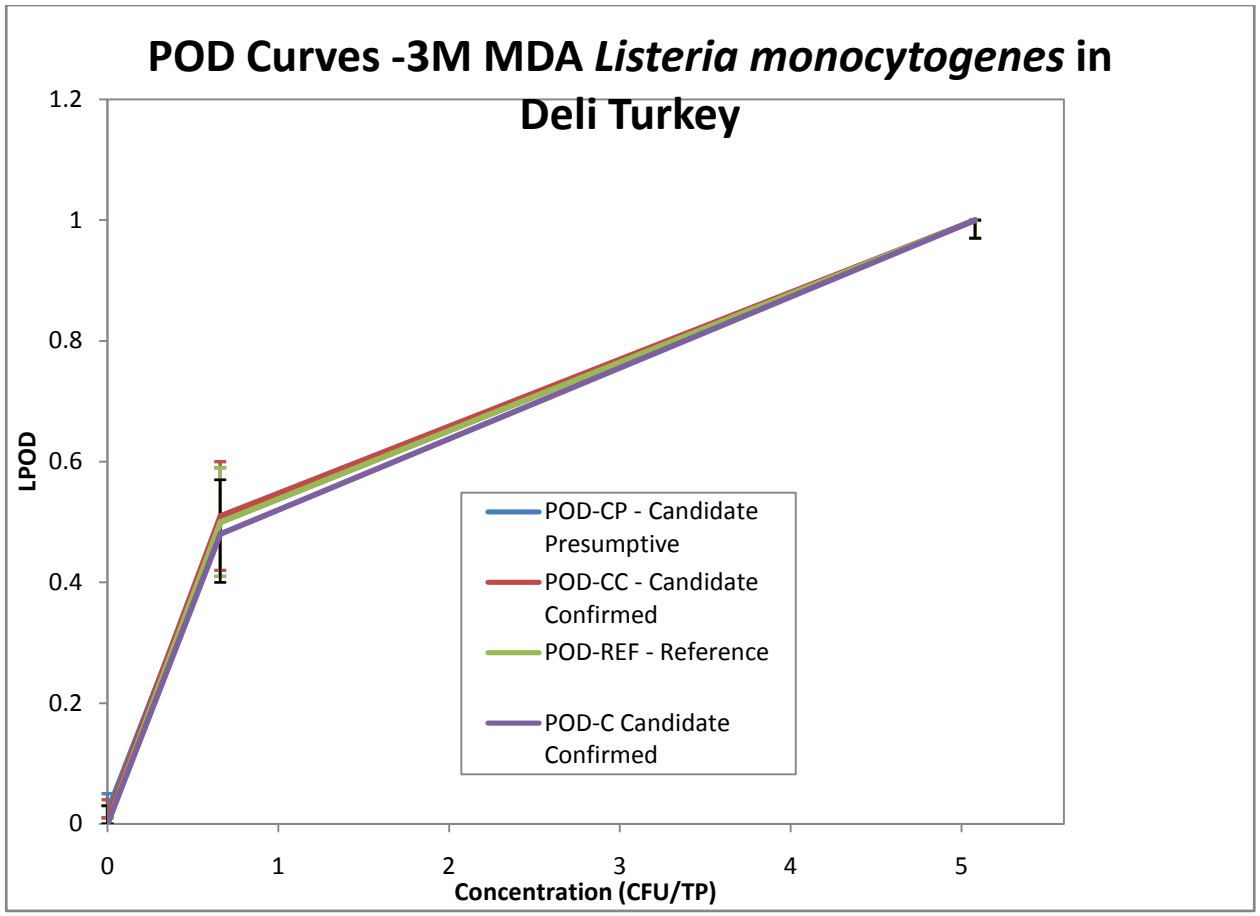
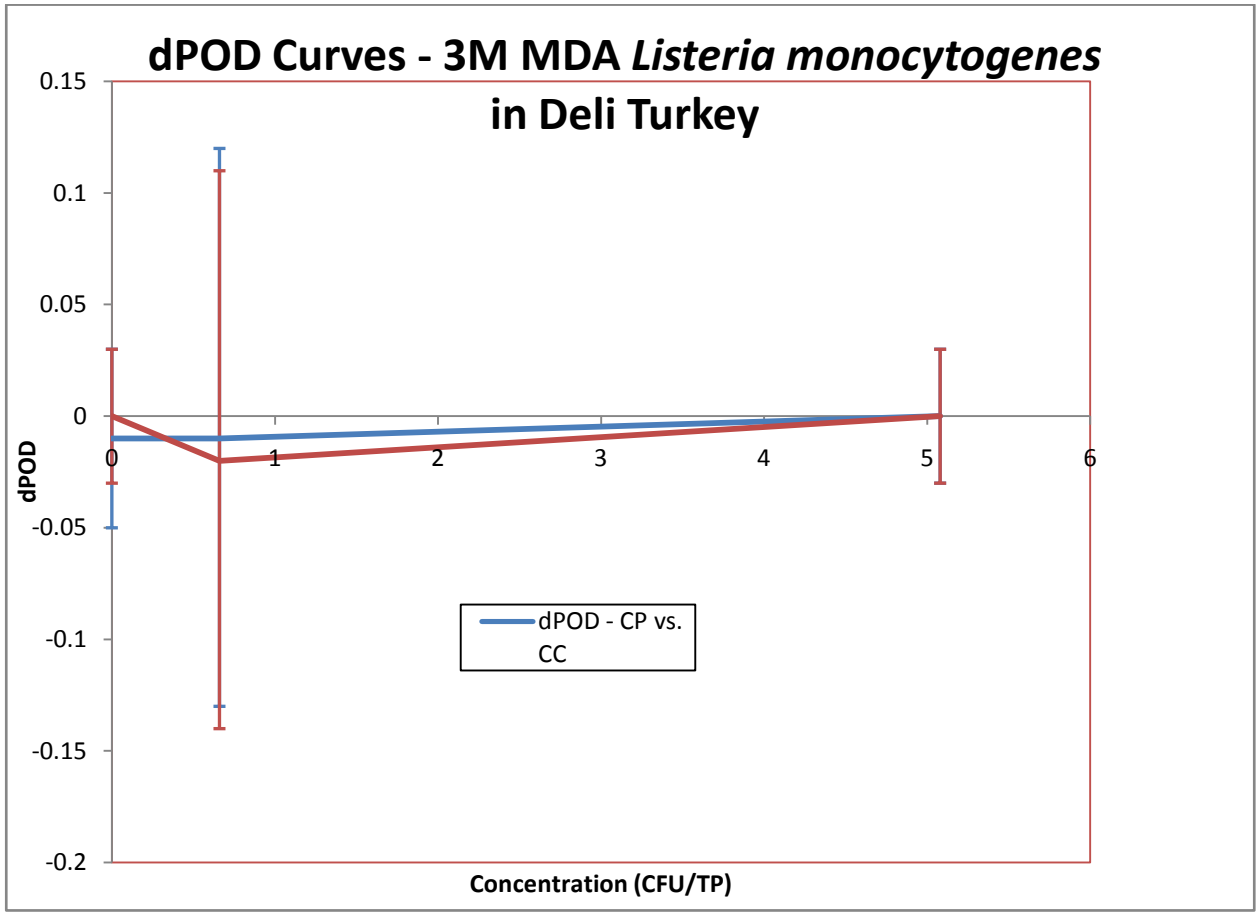

POD Curves -3M MDA *Listeria monocytogenes* in Deli Turkey
LPOD
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
3
4
5
Concentration (CFU/TP)
POD-CP - Candidate Presumptive
POD-CC - Candidate Confirmed
POD-REF - Reference
POD-C Candidate Confirmed

Figure 2B: dPOD Values of Candidate Method vs. Reference Method



1
2
3

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Official Methods of Analysis℠ (OMA)

Expert Review Panel on Microbiology for Food and Environmental Surfaces

OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final revised version of the Official Chair's Report for the Expert Review Panel on Microbiology for Food and Environmental Surfaces held on March 20, 2014.

Wendy McMahon

Wendy McMahon, Expert Review Panel Chair

April 10, 2014

Date

Please sign, date and fax this document to La'Kia Phillips at 301-924-7089.

AOAC-RI OMA ERP Chart
Chart 3-20-14 Micro

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Official Methods of Analysis[SM] (OMA)

Expert Review Panel on Microbiology for Food and Environmental Surfaces

METHODS FOR CONSIDERATION

Conclusion: The Expert Review Panel reviewed the collaborative study for OMAMAN-08: Evaluation of the 3M™ Petrifilm™ Salmonella Express System for the Detection of Salmonella in Selected Foods: Collaborative Study and Review the OMA Modification for 2013.09: 3M™ Molecular Detection Assay (MDA) Salmonella. **Methods Reviewed:** Each method collected by AOAC for consideration by this ERP is reviewed by all members. The decisions of this ERP are reflective of both the submitted method review forms and the in person meeting held on Thursday, March 20, 2014.

METHOD NO.	MANUSCRIPT TITLE
OMA Modification for 2013.09	**EVALUATION OF MODIFICATION OF THE 3M™ MOLECULAR DETECTION ASSAY (MDA) SALMONELLA FOR THE DETECTION OF SALMONELLA IN SELECTED FOODS: COLLABORATIVE STUDY** *AUTHORS* Patrick Bird, Kiel Fisher, Megan Boyle, Travis Huffman, M. Joseph Benzinger, Jr., Paige Bedinghaus, Jonathon Flannery, Erin Crowley, James Agin, David Goins, Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH 45214, DeAnn Benesh, John David, 3M Food Safety Department, 3MCenter – Bldg. 260-6B-01, St. Paul, MN 55144 *COLLABORATORS* K. Newman, V. Gill, I. Mello, M. Ontiberos, C. Gwinn, S. Moosekian, J. Marchent, J. Dyszel, M. Vross, K. Blanchard, D. Lewis, M. Horan, B. Stawick, J. Ruebl, K. Rajkowski, A. Morey, S. Montez, J. Jurgens, L. Thompson, M. Bandu, M. Oltman, D. Bosco, R. Brooks, S. Luce, H. Dammann, D. Clark Jr., W. McMahon, D. Awad, M. Kelly, M. Greenwell

ERP DECISION(S)	ERP ACTIONS FOR OTHER & FINAL ACTION REQUIREMENTS	VOTE	DECISION DATE
Motion to move forward to First Action Official Methods status.	*N/A*	MOTION PASSED ***UNANIMOUS*** *Salfinger, Hammack*	March 20, 2014

AOAC RESEARCH INSTITUTE

Official Methods of Analysis℠ (OMA)

Expert Review Panel on Microbiology for Food and Environmental Surfaces

METHOD NO.	MANUSCRIPT TITLE
OMAMAN-08	**EVALUATION OF THE 3M™PETRIFILM™SALMONELLA EXPRESS SYSTEM FOR THE DETECTION OF SALMONELLA SPECIES IN SELECTED FOODS: COLLABORATIVE STUDY** *AUTHORS* Patrick Bird, Jonathan Flannery, Erin Crowley, James Agin, David Goins, Q Laboratories, Inc., 1400 Harrison Ave, Cincinnati, OH45214, Robert Jechorek, 3M Food Safety Department, 3MCenter – Bldg. 260-6B-01, St. Paul, MN 55144 *COLLABORATORS* K. Newman, J. Pickett, J. Adams, A. Martin, J. Meyer, H. Elgaali, J. Marchant-Tambone, K. Blanchard, D. Lewis, R. Colvin, B. Stawick, K. Rajkowski, D. Rodgers, K. Beers, A. Morris, K. McCallum, A. Morey, W. Fedio, R. Brooks, M. Boyle

ERP DECISION(S)	ERP ACTIONS FOR OTHER & FINAL ACTION REQUIREMENTS	VOTE	DECISION DATE
Motion to move forward to First Action Official Methods status.	*N/A*	MOTION PASSED ***UNANIMOUS*** *Brodsky, Douey*	March 20, 2014

AOAC RESEARCH INSTITUTE

Official Methods of Analysis℠ (OMA)

Expert Review Panel on Microbiology for Food and Environmental Surfaces

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Michael Brodsky, Brodsky Consultants
Maya Achen, Ohio Dept. of Agriculture
Donna Douey, CFIA
Maria C. Fernandez, ANMAT/Ministry of Health Univ. of Buenos Aires
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Tony Hitchins, US FDA-CFSAN (retired)
Yvonne Salfinger

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Yi Chen, US FDA-CFSAN (Alternate)

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Yvonne Salfinger	Safety Reviewer
Caryn Thompson	Statistical Reviewer

Expert Review Panel
Microbiology for Food and Environmental Surfaces
OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT

ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final version of the Official Chair's Report for the Expert Review Panel for Microbiology for Food and Environmental Surfaces was held on Wednesday, December 10, 2014 at AOAC INTERNATIONAL Headquarters located at 2275 Research Blvd, Rockville, Maryland 20850.

WENDY MCMAHON, SILLIKER LABORATORIES
Expert Review Panel Co-Chair

MICHAEL BRODSKY, BRODSKY CONSULTING
Expert Review Panel Co-Chair

Wendy McMahon
Date Jan 23, 2015

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Contact:
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Deborah McKenzie, Sr. Director, DMcKenzie@aoac.org

EXPERT REVIEW PANEL MEETING ATTENDEES

Expert Review Panel Chair (s)
Michael Brodsky, Brodsky Consulting
Wendy McMahon, Silliker Laboratories

Expert Review Panel Members
Patrice Arbault, BioAdvantage
Yi Chen, FDA/ Tom Hammack, FDA
Maria Christina Fernandez, University of Buenos Aires
Tony Hitchins, FDA/CFSAN (Retired)
Sam Mohajer, Canadian Food Inspection Agency
Paul Wehling, General Mills

Method Authors
DeAnn Benesh, 3M Food Safety
Robert Jechorek, 3M Food Safety
Lisa Montereso, 3M Food Safety
Erin Crowley, Q Labs
Patrick Bird, Q Labs

AOAC Staff
Jim Bradford, Executive Director
Deborah McKenzie
Tien Milor
La'Kia Phillips

Observers
N/A

EXPERT REVIEW PANEL, METHOD BACKGROUND, AND CONCLUSIONS

Criteria for Vetting Methods to be considered:
AOAC convened the *Official Methods of Analysis*℠ (OMA) Expert Review Panel for Microbiology for Foods and Environmental Surfaces on Wednesday, December 10, 2014 from 8:00am to 12:00pm at AOAC INTERNATIONAL Headquarters located at 2275 Research Blvd, Rockville, Maryland 20850.

The purpose of the meeting was to review and evaluate OMAMAN-16: 3M™ Petrifilm™ Rapid Yeast and Mold Collaborative Study submitted by Bob Jechorek of 3M Food Safety located at 3M Center, Building 260-06-B-01, St. Paul, MN 55144 and Erin Crowley, Q Laboratories, 1400 Harrison Avenue, Cincinnati, OH 45214. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, method safety checklist, AOAC *Performance Tested Methods*℠ validation report, *Performance Tested Methods*℠ protocol and package insert.

OMAMAN-17: Evaluation Of The 3M™ Molecular Detection Assay (MDA) Listeria for the Detection of Listeria Species submitted by Lisa Monteroso of 3M Food Safety Department located at 3M Center, Building 0260-06-B-01, Saint Paul, Minnesota 55144-1000 U.S.A. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, method safety checklist, AOAC *Performance Tested Methods*℠ validation report, two (2) *Performance Tested Methods*℠ modification reports and package insert.

OMAMAN-18: Evaluation Of The 3M™ Molecular Detection Assay (MDA) Listeria Monocytogenes for the Detection of Listeria Monocytogenes submitted by Lisa Monteroso of 3M Food Safety Department located at 3M Center, Building 0260-06-B-01, Saint Paul, Minnesota 55144-1000 U.S.A. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, method safety checklist, AOAC *Performance Tested Methods*℠ validation report, and package insert.

Criteria for Vetting Experts and Selection Process:
The following seven (7) candidates and one (1) alternate member were submitted for consideration by the Official Methods Board to evaluate candidate methods for Pesticide Residues methods as per the Expert Review Panel (ERP) Policies and Procedures. The candidates were highly recommended by the Chemical Contaminants and Residues in Foods Community, have participated in various AOAC activities, including but limited to, Method Centric Committees that were formed under the legacy OMA pathway, and were vetted by the Official Methods Board. The experts are Amy Brown, Jo Marie Cook (Alternate), Julie Kowalski, John Reuther, Marina Torres, Jian Wang, and Xiaoyan Wang.

ERP Orientation:
The ERP members have completed the mandatory AOAC Expert Review Panel Orientation Webinar on Wednesday, November 5, 2014.

Expert Review Panel Meeting Quorum
The meeting of the Expert Review Panel was held in person. A quorum is the presence of seven (7) members or 2/3 of the total vetted ERP, whichever is greater. Eight (8) out of the eleven (11) voting members were present and therefore met a quorum to conduct the meeting. It was also noted that Jim Agin who was not present, will not participate on this Expert Review Panel.

Standard Method Performance Requirements (SMPRs): N/A

Conclusion:
The Expert Review Panel reviewed OMAMAN-16: 3M™ Petrifilm™ Rapid Yeast and Mold, OMAMAN-17: Evaluation Of The 3M™ Molecular Detection Assay (MDA) Listeria For The Detection Of Listeria Species, and OMAMAN-18: Evaluation Of The 3M™ Molecular Detection Assay (MDA) Listeria Monocytogenes For The Detection Of Listeria Monocytogenes and have adopted these methods for AOAC First Action Official Method status by unanimous decision as noted in the meeting minutes.

Subsequent ERP Activities:
ERP members will continue to evaluate the method for 2 years.

MEETING MINUTES

I. Welcome and Introductions

The Expert Review Panel Co-chairs, Michael Brodsky and Wendy McMahon welcomed Expert Review Panel members, initiated introductions, and discussed with the panel the goal of the meeting.

II. Review of AOAC Volunteer Policies

A brief overview of AOAC Volunteer Policies, Volunteer Acceptance Agreement and and Expert Review Panel Policies and Procedures which included Volunteer Conflicts of Interest, Policy on the Use of the Association, Name, Initials, Identifying Insignia, Letterhead, and Business Cards, Antitrust Policy Statement and Guidelines, and the Volunteer Acceptance Form (VAF). All members of the ERP were required to submit and sign the Volunteer Acceptance Form.

III. Expert Review Panel Process Overview and Guidelines

Deborah McKenzie presented a quick overview of the Expert Review panel process including meeting logistics, consensus, First Action to Final Action requirements, and documentation.

IV. Review of Methods

All ERP members presented a review and discussed the proposed collaborative study manuscript for The purpose of the meeting was to review and evaluate OMAMAN-16: 3M™ Petrifilm™ Rapid Yeast And Mold Collaborative Study. The method authors Robert Jechorek of 3M Food Safety and Erin Crowley of Q Laboratories were both present and able to address questions and concerns of the ERP members. A summary of comments was provided to the ERP members.[1]

MOTION:
Motion by Brodsky; Second by McMahon, for the method to move forward for First Action Official Method Status.
Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous). *Motion Passed.*

All ERP members presented a review and discussed the proposed collaborative study manuscript for The purpose of the meeting was to review and evaluate OMAMAN-17: Evaluation Of The 3M™ Molecular Detection Assay (MDA) Listeria For The Detection Of Listeria Species. The method authors Lisa Montereso of 3M Food Safety and Erin Crowley of Q Laboratories were both present and able to address questions and concerns of the ERP members. A summary of comments was provided to the ERP members.[2]

MOTION:
Motion by Brodsky; Second by Arbault, for the method to move forward for First Action Official Method Status.
Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous). *Motion Passed.*

All ERP members presented a review and discussed the proposed collaborative study manuscript for The purpose of the meeting was to review and evaluate OMAMAN-18: Evaluation Of The 3M™ Molecular Detection Assay (MDA) Listeria Monocytogenes for The Detection Of Listeria Monocytogenes. The method authors Lisa Montereso of 3M Food Safety and Erin Crowley of Q Laboratories were both present and able to

[1] Attachment 1: Summary of Expert Reviewer Comments for OMAMAN-16
[2] Attachment 2: Summary of Expert Reviewer Comments for OMAMAN-17

address questions and concerns of the ERP members. A summary of comments was provided to the ERP members.[3]

MOTION:
Motion by Hitchins, Second by Mohajer, for the method to move forward for First Action Official Method Status.
Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous). ***Motion Passed.***

V. Discuss Final Action Requirements for First Action Official Methods (if applicable)
No further action was discussed at this time.

VI. Adjournment

[3] Attachment 3: Summary of Expert Reviewer Comments for OMAMAN-18

TECHNICAL EVALUATION CRITERIA Is the test kit method scientifically and technically sound?	
ER 1	yes
ER 2	yes
ER 3	yes
ER 4	yes
ER 5	yes
ER 6	yes
ER 7	yes
ER 8	yes
Have sufficient controls been used, including those required to calculate the rate of false-positive and false-negative results where appropriate?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Is sufficient information included for system suitability determination and product performance or acceptance testing?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	
ER 6	Yes
ER 7	Yes
ER 8	Yes
Are the conclusions statements valid based upon data presented?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	No
ER 8	No

Do you agree that the evidence or data from this and previous studies support the proposed applicability statement?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Are there sufficient data points per product evaluated in accordance with AOAC requirements?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
General Comments about the Method Scope/Applicability:	
ER 1	The method scope and applicability are clearly described, and the Petrifilm protocols have been clearly presented. Both incubation temperature options were also clearly stipulated. Description of the look like colonies were sufficiently explained.
ER 2	A statement about growth curve for the yeast selected was made on page 3, row 14-15. This culture incubated for 48h while there are strains requiring 5-7 days incubation. The method includes up to 60 h incubation. Reviewer recommends applicability statement to this point. The background section could include this point and then the discussion could address it.
ER 3	Almonds and Ground Beef have been used for the collaborative study which are both appropriate matrices and were used in the pre-collaborative study.
ER 4	No additional comments
ER 5	The method is useful for rapid quantification of spoilage microorganisms
ER 6	Accurately reflects breadth and depth of study
ER 7	None
ER 8	1. Page 5, lines 27-29, Sterile Diluent. The method lists several diluents that can be used with this method, including 0.1% peptone water. All of the diluents, other than 0.1% peptone water, should be removed from the method, since the method was validated with 0.1 peptone water and no other diluent. The implied conclusion is that the method, as given by the draft official method, is that it can be run with any diluent even though it was only validated with 0.1 % peptone water. 2. Page 5, lines, 7 - 9, applicability (here and elsewhere in the report). The method refers to yogurt, ready-made-pie, frozen ground beef patties, sandwiches, and dehydrated soup. These are not specific enough. Yogurt and beef should have fat percentages. The types of pie, sandwiches and dehydrated soup should be specified. For example, apple pie, chicken soup, tuna sandwich... The applicability statement is too broad as written.

General comments about the method:	
ER 1	The method is very well described in the various documents.
ER 2	NA
ER 3	The method is much simpler compared to traditional method and the space savings in the use of Petrifilm are significant as compared to traditional agar plates.
ER 4	No additional comments
ER 5	The method is really useful due to it significantly reduces test times
ER 6	Well written and easy to follow
ER 7	none
ER 8	1. Page 7, line 17. There should be references directing the analyst to methods for the further identification of yeast/fungal isolates. They should be ISO, BAM, MLG and others.
Pros/Strengths of the Manuscript:	
ER 1	Good description of the sample preparation protocols. Efficient description of the collab study workflow and organization. Tables are very useful for summarizing the results.
ER 2	Very well written.
ER 3	The Manuscript is well written and the information flow is in an understandable order.
ER 4	Generally well written.
ER 5	It is a simple method for working
ER 6	Very thoroughly written and detailed
ER 7	Well written in general.
ER 8	It is well written.
Cons/Weaknesses of the Manuscript:	
ER 1	Very minor edits: page 7, line 25, reports lab 5 as one of the 4 labs with deviations, but in table 1 page 13, lab 6 is marked as the lab showing deviations for ground beef??? Reading through the report and the pack insert, it remains unclear if the minimum incubation is 48 hours or 46 hours since it is stipulated that incubation shall be 48+/-2 hours but reading is required at 48 hours: is minimal time of 48 hours of incubation is required?
ER 2	NA
ER 3	Table 1 describes which data sets were not used in the statistical analysis; however, there are not indications as such in tables 2 – 9 where raw data is presented. It may help to identify the labs who's data sets were excluded in each table using a superscript letter.
ER 4	Need to elaborate on the issues and possible causes of those issues of laboratories whose data were not used in the study.
ER 5	no
ER 6	None
ER 7	Page 1, line 24 states "unpaired study design" but page 4 line, line 22 states "paired study design". Please clarify in the manuscript. Page 3, line 29: Clarify that after lyophilization dilutions were done with sterile NFDM powder or reconstituted NFDM. Page 4, line 38: Increase font size. Page 4, line47: Justify or omit reverse transformed mean difference here and in Tables 2014.1 and 2014.2.[Continued] Page 7, line 24: Insert "valid" before both "data" words. Page 7, line 33: Omit Figs 1-4 which are somewhat redundant. Add statement about acceptability of Youden plots. Page 7, line 37: Remove "reverse transformed difference" here and in Tables 2014.1 &.2. Page 8, lines 31-33: State the repeatability SD values supporting this assertion. Page 8, lines 44-46: State the repeatability SD values supporting this assertion.

	Page 9, lines 8-10: State the repeatability SD values supporting this assertion. Page 9, lines 30-32: State the repeatability SD values supporting this assertion. Page 9, lines 42-44: State the repeatability SD values supporting this assertion. Page 10, lines 7-9: State the repeatability SD values supporting this assertion. Page 10, lines 21-23: State the repeatability SD values supporting this assertion. Page 10 lines 42-44: State the repeatability SD values supporting this assertion. Add the requested repeatability SD values as footnotes to relevant Tables.
ER 8	Applicability statement is too general. Tables are not properly footnoted.
Supporting Data and Information: Does data from collaborative study support the method as written?	
ER 1	Yes.
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	yes
ER 6	Yes
ER 7	Yes, in general.
ER 8	Yes.
Supporting Data and information: Does data collected support the criteria given in the collaborative study protocol?	
ER 1	The data supports the criteria.
ER 2	Yes
ER 3	No
ER 4	yes
ER 5	yes
ER 6	Yes
ER 7	Yes.
ER 8	Yes.
Are there any concerns regarding the safety of the method?	
ER 1	To be covered once Safety Advisor review is presented.
ER 2	No
ER 3	No
ER 4	No
ER 5	No
ER 6	No
ER 7	No.
ER 8	No.
Are there any concerns regarding the data manipulation, data tables, or statistical analysis?	
ER 1	No concerns.
ER 2	No
ER 3	The manuscript would be much stronger if a more detail rational is given as to why some of the collaborative labs were excluded. It is understandable to have such occurrences in a large collaborative study.
ER 4	No
ER 5	No
ER 6	No

ER 7	In Table 2014.1 for the RYM method it is not clear what data from Tables 2, 3, 4 & 5 is used to get the SD values for repeatability and reproducibility. In Table 2014.2 for the RYM method it is not clear what data from Tables 6 7, 8 & 9 is used to get the SD values presented for repeatability and reproducibility. Typically the SD for reproducibility is noticeably larger than that for repeatability. This is true for the 2014.2 (almond) results by the RYM and the FDA/ISO methods. However, it is not so for the 2014.1 (beef) results by the RYM or reference method. So perhaps the calculations and data inputs should be checked. [Continued] In many cases, especially in Table 2014.1 the mean RYM and reference methods' mean counts are not significantly different and yet the differences, although not very different, are often significantly different. In Table 2014.1 perhaps this is related to the fact that only 11 collaborators provided valid results.
ER 8	None.
General Comments (2)	
ER 1	
ER 2	NA
ER 3	NA
ER 4	No additional comments
ER 5	
ER 6	
ER 7	None
ER 8	Tables 2014.1 & 2014.2. It should be footnoted that the BAM and ISO methods are identical when using 0.1 % peptone water. as was done in this collaborative study.
EDITORIAL EVALUATION CRITERIA Is the Validation Study Manuscript in a format acceptable to AOAC?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Is the method described in sufficient detail so that it is relatively easy to understand, including equations and procedures for calculation of results (are all terms explained)?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	No
ER 8	Yes
Are the figures and tables sufficiently explanatory without the need to refer to the text?	
ER 1	Yes

ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	No
Are all the figures and tables pertinent?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	No
ER 8	Yes
Could some be omitted and covered by a simple statement?	
ER 1	No
ER 2	No
ER 3	No
ER 4	No
ER 5	No
ER 6	Yes
ER 7	Yes
ER 8	No
Are the references complete and correctly annotated?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Does the method contain adequate safety precaution reference and/or statements?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes

ER 8	Yes
RECOMMENDATION: **Do you recommend that the ERP adopt this method as an AOAC *Official Methods of Analysis (First Action status)*?**	
ER 1	Yes.
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	yes
ER 6	Yes
ER 7	This method is potentially recommendable for adoption after manuscript clarifications are made.
ER 8	Yes, if my above comments regarding applicability and diluent are addressed. The package insert also needs to be revised in light of these comments. If multiple diluents are recommended, even though only 1 was validated, then I would recommend against giving the method First Action Status.
AFTER FIRST ACTION STATUS: **Is there any additional information that the ERP should consider in order to recommend the method for Final Action status?**	
ER 1	No.
ER 2	NA
ER 3	NA
ER 4	No additional comments
ER 5	No
ER 6	User comments
ER 7	No
ER 8	Two years worth of use in the field, without substantial problems, should be the criteria for making this a final action method.
General Comments (3)	
ER 1	
ER 2	NA
ER 3	NA
ER 4	No additional comments
ER 5	
ER 6	
ER 7	None
ER 8	1. Package insert, page 4 of 8, Sterile Diluent. The method lists several diluents that can be used with this method, including 0.1% peptone water. All of the diluents, other than 0.1% peptone water, should be removed from the method, since the method was validated with 0.1 peptone water and no other diluent. 2. Package insert, page 6 of 8, applicability. The method refers to yogurt, ready-made-pie, frozen ground beef patties, sandwiches, and dehydrated soup. These are not specific enough. Yogurt and beef should have fat percentages. The types of pie, sandwiches and dehydrated soup should be specified. For example, apple pie, chicken soup, tuna sandwich... The applicability is too broad as written.

TECHNICAL EVALUATION CRITERIA Is the test kit method scientifically and technically sound?	
ER 1	yes
ER 2	yes
ER 3	yes
ER 4	yes
ER 5	yes
ER 6	yes
ER 7	yes
ER 8	yes
Have sufficient controls been used, including those required to calculate the rate of false-positive and false-negative results where appropriate?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Is sufficient information included for system suitability determination and product performance or acceptance testing?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	No
ER 8	Yes
Are the conclusions statements valid based upon data presented?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	No
ER 5	Yes
ER 6	Yes
ER 7	No
ER 8	Yes

Do you agree that the evidence or data from this and previous studies support the proposed applicability statement?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	No
ER 6	Yes
ER 7	No
ER 8	Yes
Are there sufficient data points per product evaluated in accordance with AOAC requirements?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	No
ER 6	Yes
ER 7	No
ER 8	
General Comments about the Method Scope/Applicability:	
ER 1	Method is applicable to a wide variety of foods judging from the PTM study.
ER 2	NA
ER 3	No additional comments
ER 4	Cottage cheese has been used for this collaborative study which is an appropriate matrix since it was also used in the pre-collaborative study.
ER 5	The method scope and applicability are clearly described, and the various enrichment protocols have been extensively presented and summarized in tables. The possibility to use different enrichment media, with one or two enrichment steps according to the matrix, makes the overall analysis more complex, but all information can be found in the document. Nonetheless, no indication has been provided regarding the choice of mLRB versus Demi-Fraser for the user: does it matter?
ER 6	Listeria is a bacterium frequently isolated from different types of food and surfaces. This method allows to detect Listeria in an easy and rapid way with a high sensitivity and specificity.
ER 7	Overall, the method looks good. My main question concerns the application of the guidelines (Appendix J, section 4.3.4, "Matrix Selection"), which affects applicability. Only Demi-Fraser broth was used in the collaborative study, but the official method calls for both Demi-Fraser and mLRB. Section 4.3.4 states "For methods with more than one sample preparation/enrichment, one matrix per procedure may be required in the collaborative study. The determination if the procedures differ significantly to warrant expanding the collaborative study is made by the appropriate method volunteer(s) in consultation with the Study Director." Was a decision reached between the study director and volunteers? If not, then it seems to me that the method may only be validated for use with Demi-Fraser broth.
ER 8	Appropriate

General comments about the method:	
ER 1	Clearly the isothermal feature of the method is very convenient. It would be nice if a few sentences were added outlining the biochemical/molecular basis of the method in general terms.
ER 2	Page 3, row 21-22, Remove shipped to collaborators as it is described at row 35 along with randomizing and blind coding.
ER 3	No additional comments
ER 4	The method appears to be a very rapid method with a simple work flow that allows for the rapid detection of Listeria species.
ER 5	The method is very well described in the various documents , and the various steps of the sample preparation and MDA assay are exhaustively presented, including the ciritical steps. The method can be applied to a large variety of matrices covering different food categories and environmental surfaces. Enrichment media and procedures may vary according to the matrix, and therefore, users shall be clearly informed about the enrichment conditions for given matrices. Enrichment times usually mention a upper limit of incubation: what does happen if the user exceeds this upper value?
ER 6	This is a method very useful for the industry and it allows more short times for detection of Listeria than with culture methods . So, this is very useful for facilitate regional trade.
ER 7	None.
ER 8	Direct and easy to follow
Pros/Strengths of the Manuscript:	
ER 1	Generally a very clearly written manuscript.
ER 2	Very well written.
ER 3	Generally well written.
ER 4	Manuscript is well written and the information flow is in an understandable order.
ER 5	Exhaustive presentation of the various AOAC-PTM validations/extensions. Complete description of the sample preparation protocols. Efficient description of the collab study workflow and organization. Tables are very useful for summarizing the various enrichment protocols of the method, for presenting the results.
ER 6	The method has a high sensitivity and specificity.
ER 7	Well written.
ER 8	Well written with full details

Cons/Weaknesses of the Manuscript:	
ER 1	Some editorial suggestions are made below.
ER 2	NA
ER 3	Need to elaborate on the issues and possible causes of those issues of laboratories whose data were not used in the study.
ER 4	• Correction on table 2 page 17, AOAC OMA 993.12, lab 6 is excluded in the analysis and should have a superscript b. • It is unclear as to why the reference method chosen is not the BAM reference method which would be in this reviewer's opinion a better and more comprehensive method to use. While the reference method used is an OMA method, the use of the BAM method would have given more confidence to regulatory bodies reviewing this method. • The uses of positive and negative controls are not well defined. • There may be some value in stating the ISO status of the testing labs to add more confidence on the results. • The reasons for the exclusion of labs 6 and 13 may need more detail. In both cases the data set suggests that these labs were simply excluded since they detected false negative results which I am sure is not the case; however, no detail scientific explanation is provided. • While many of the acronyms used in the data and statistical tables are well known and are described in the Appendix J of the AOAC method validation guidelines, it may be beneficial to include a section on the explanation of these terms. Since this is my first time reviewing such a manuscript, I am not sure if these were included in the past but they may help some understand the tables.
ER 5	The manuscript does not discuss the reasons behind the selection of DF broth enrichment protocol for the collaborative study: mLRB vs DF? 1 step enrichment vs 2 step enrichment? There are very few mistakes in the report among which one is more relevant: table 2 presenting individual collaborator results seem wrong for the low level test portion results as the total from the table give 67 positive when the report mentions 73. Comparing data with table 2014.2A (p21), it seems that numbers for labs 2 and 8 are wrong in table 2: is that right? Please advise.
ER 6	NO
ER 7	None observed.
ER 8	None
Supporting Data and Information: Does data from collaborative study support the method as written?	
ER 1	Yes.
ER 2	Yes
ER 3	Yes
ER 4	Since the cottage cheese is a posturized product, a heat stress inoculum would have been more appropriate; however, this was also not done during the pre-collaborative study.
ER 5	Yes & No. Yes when it relates to the DF-1 step enrichment protocol. No because mLRB and DF-2 step enrichment protocols have not been evaluated during this collaborative study. That is my understanding that each of the different enrichment protocols must be evaluated during a collaborative study to be submitted to OMA first action: am I mistaken? Some of the claimed matrices require a 2-step enrichment protocol (bagged raw spinach and whole cantaloupe)
ER 6	yes
ER 7	I marked "no" for questions 3 - 6 above, because Tables A and B make "claims" that are not supported by the PTM or collaborative studies. Both tables have a section for "other matrices" that include dairy, vegetables, meat, poultry, seafood, and fruit, but none of these categories have been fully validated (only 1 or 2 matrices per category; traditionally, you need at least 3 matrices to claim a category). Please remove the "For Other Matrices" sections from both Tables A and B, so that the Tables will be aligned

	with the applicability statement.
ER 8	Yes
Supporting Data and information: Does data collected support the criteria given in the collaborative study protocol?	
ER 1	Yes.
ER 2	Yes
ER 3	yes
ER 4	YES, the data has been generated in accordance to the collaborative study protocol.
ER 5	The data supports the criteria regarding the use of DF broth and one step enrichment protocol.
ER 6	yes
ER 7	Please remove the "For Other Matrices" sections from both Tables A and B, so that the Tables will be aligned with the applicability statement (see above).
ER 8	Yes
Are there any concerns regarding the safety of the method?	
ER 1	No
ER 2	No
ER 3	No
ER 4	No
ER 5	To be covered once Safety Advisor review is presented.
ER 6	No
ER 7	No.
ER 8	No
Are there any concerns regarding the data manipulation, data tables, or statistical analysis?	
ER 1	No
ER 2	No
ER 3	No
ER 4	I would like to bring the following points for discussion by the group: • While the AOAC guidelines under Appendix J, Annex F prescribe the analysis of the data set as a whole for the POD analysis, is there a need to also consider the equivalency of the methods in each individual lab. This may be outside of the realm of the guidelines, but I believe that this type of evaluation could be useful. For example in this method, overall the combined data set produced 66 and 64 positive results for the presumptive and confirmed candidate results respectively, while a total of 73 positives were detected using the reference method. As a whole in this case the LCL and the UCL of the dPOD encompasses the 0 value showing no statistical differences. This is however not true for two of the participating laboratories,laboratory 7 and 9 both produced differences between the candidate and the reference method which are statistically significant. In both cases the candidate method produced results that were much lower than the reference method. Does this indicate that there was a particular problem with these sample sets, the method or the analysis? The table below shows an example of the results in lab 7 where the candidate method detected 4 confirmed positives whereas the reference method detected 10. • It is interesting to see that sample 9 in the low sample set of the candidate method for labs 7-15 all produced negative results and overall only one lab that in the included sample set produced a positive sample. To illustrate this point, 1 out of 11 labs had a positive sample giving a POD of 9%. This is much lower than the overall POD of about 50% for the sample set and falls outside of the statistical (normal) distribution expected with a 50 % fractional positive result. All other sample sets exhibit a more normal distribution of fractional positive results as would be expected. The same pattern does not repeat anywhere in the reference

	method data set. • The APC counts for the sample sets seem to have a broad range of values where the range covers 3 logs of counts. I was not expecting such a difference given the method that is used to enumerate these results is a well validated and used quantitative method.
ER 5	No concerns.
ER 6	No
ER 7	No.
ER 8	No
General Comments (2)	
ER 1	None.
ER 2	NA
ER 3	No additional comments
ER 4	NA
ER 5	
ER 6	
ER 7	None.
ER 8	
EDITORIAL EVALUATION CRITERIA Is the Validation Study Manuscript in a format acceptable to AOAC?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Is the method described in sufficient detail so that it is relatively easy to understand, including equations and procedures for calculation of results (are all terms explained)?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes

Are the figures and tables sufficiently explanatory without the need to refer to the text?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	No
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Are all the figures and tables pertinent?	
ER 1	No
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Could some be omitted and covered by a simple statement?	
ER 1	Yes
ER 2	No
ER 3	No
ER 4	
ER 5	No
ER 6	No
ER 7	No
ER 8	Yes
Are the references complete and correctly annotated?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes

Does the method contain adequate safety precaution reference and/or statements?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
General Comments (3)	
ER 1	1.Page 19 is titled Appendix. What is the intent here? 1. An appendix with data? 2. Appended data is not to be published? 2. Figures: omit as they are repetitious. 3. Page 1, line 44: Replace "climates" with "conditions". 4. Page 1, Line 44: Define "high pH" and provide reference. [CONTINUED] Is this an allusion to resistance to alkaline sanitizers or to the high end of the pH 4.3-9.4 growth range of listeria? Page 1, lines 40 & 42: Change "Listeriosis" to "listeriosis". Page 2, line 33: Insert "the reproducibility among different laboratories of" between "compare" and "the". Page3, lines 24-26: Sentence is awkward - restructure. Page 4, lines 3 and 4:Change "(12 high, 12 low and 12 controls for each method)" to "(12 high inoculum, 12 low inoculum and 12 uninoculated controls for each method)". Page 5, line 12: "Appendix"? See comment above. Page 14, lines 42-43: Provide website address. Page 18, Table 2014.1A: Readability of the table could be enhanced by delineating the 4 subsections. For example, one could add blank lines between the Candidate Presumptive, Candidate Confirmed, Positive Reference and dLPOD subsections. Pages 21-22, Table 2014.2A: Suggest making this into 3 tables.
ER 2	NA
ER 3	No additional comments
ER 4	
ER 5	Few edits to be covered. Will be discussed during the meeting.
ER 6	On Page 12 line 30 “to the” is mentioned twice
ER 7	None.
ER 8	

RECOMMENDATION: **Do you recommend that the ERP adopt this method as an AOAC *Official Methods of Analysis (First Action status)*?**	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	YES, while some issues may require discussion, the method has proven its performance.
ER 5	Yes but limited to DF and the one step enrichment protocol. Therefore, matrix claim shall be narrowed. To be discussed during the ERP meeting.
ER 6	Yes
ER 7	Yes, as long as Tables A & B are corrected as specified above.
ER 8	Yes
AFTER FIRST ACTION STATUS: **Is there any additional information that the ERP should consider in order to recommend the method for Final Action status?**	
ER 1	Why was only one food collaboratively studied?
ER 2	NA
ER 3	No additional comments
ER 4	NA
ER 5	Recommendation to monitor the false positive rates generated by the method.
ER 6	No
ER 7	None, other than 2 years of field use.
ER 8	User feedback

TECHNICAL EVALUATION CRITERIA Is the test kit method scientifically and technically sound?	
ER 1	yes
ER 2	yes
ER 3	yes
ER 4	yes
ER 5	yes
ER 6	yes
ER 7	yes
ER 8	yes
Have sufficient controls been used, including those required to calculate the rate of false-positive and false-negative results where appropriate?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Is sufficient information included for system suitability determination and product performance or acceptance testing?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Are the conclusions statements valid based upon data presented?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	No
ER 6	No
ER 7	Yes
ER 8	Yes

Do you agree that the evidence or data from this and previous studies support the proposed applicability statement?	
ER 1	No
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	No
ER 7	Yes
ER 8	Yes
Are there sufficient data points per product evaluated in accordance with AOAC requirements?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	No
ER 7	Yes
ER 8	Yes
General Comments about the Method Scope/Applicability:	
ER 1	Table A, pp 8 & 9 of the report, should not have an entry entitled "For Other Matrices", since "other matrices" were not validated in either the pre-collaborative (PTM) or collaborative studies. It should only include validated matrices. Table A, in its current form, implies that the applicability is broader than is substantiated by the validation study.
ER 2	This isothermal nucleic acid amplification method detects L. monocytogenes in a variety of foods and on a variety of environmental surfaces found in food processing plants.
ER 3	NA
ER 4	No additional comments
ER 5	Cottage cheese and deli turkey have been used for the collaborative study which are appropriate matrices and were both used in the pre-collaborative study.
ER 6	The method scope and applicability are clearly described, and the enrichment protocols have been properly presented and summarized in a table. The possibility to use one or two enrichment steps according to the matrix, makes the overall analysis more complex, but all information can be found in the document.
ER 7	Listeria is a bacterium frequently isolated from different types of food and surfaces. This method allows to detect Listeria in an easy and rapid way with a high sensitivity and specificity.
ER 8	Accurately reflects breadth and depth of PTM and collaborative studies

General comments about the method:	
ER 1	None.
ER 2	The inter-laboratory reproducibility of the method is evaluated in two foods, cottage cheese and deli turkey. Heat stressed cells of L. monocytogenes were used for the latter matrix.
ER 3	Page 3, row 27-28, Remove shipped to collaborators as it is described at page 4 row 1 along with randomizing and blind coding.
ER 4	No additional comments
ER 5	The method appears to be a very rapid method with a simple work flow that allows for the rapid detection of Listeria monocytogenes.
ER 6	The method is very well described in the various documents , and the various steps of the sample preparation and MDA assay are exhaustively presented, including the ciritical steps. The method can be applied to a large variety of matrices covering different food categories and environmental surfaces. Enrichment protocol may vary according to the matrix, and therefore, users shall be clearly informed about the enrichment conditions for given matrices. Enrichment times usually mention a upper limit of incubation: what does happen if the user exceeds this upper value?
ER 7	This is a method very useful for the industry and it allows more short times for detection of Listeria than with culture methods . So, this is very useful for facilitate regional trade.
ER 8	Well conceived and scientifically sound
Pros/Strengths of the Manuscript:	
ER 1	Well written.
ER 2	The validation is generally clearly described.
ER 3	Very well written.
ER 4	Generally well written.
ER 5	Manuscript is well written and the information flow is in an understandable order.
ER 6	Exhaustive presentation of the various AOAC-PTM validations/extensions. Complete description of the sample preparation protocols. Efficient description of the collab study workflow and organization. Tables are very useful for summarizing the various enrichment protocols of the method, for presenting the results.
ER 7	The method has a high sensitivity and specificity.
ER 8	Clearly written and explained

Cons/Weaknesses of the Manuscript:	
ER 1	None.
ER 2	The manuscript is lengthy.
ER 3	NA
ER 4	Need to elaborate on the issues and possible causes of those issues of laboratories whose data were not used in the study.
ER 5	• Correction on table 2 page 19 for cottage cheese matrix, AOAC OMA 993.12, lab 6 is excluded in the analysis and should have a superscript b. • It is unclear as to why the reference method chosen is not the BAM reference method which would be in this reviewer's opinion a better and more comprehensive method to use. While the reference method used is an OMA method, the use of the BAM method would have given more confidence to regulatory bodies reviewing this method. • The uses of positive and negative controls are not well defined. • There may be some value in stating the ISO status of the testing labs to add more confidence on the results. • The reasons for the exclusion of labs 6 and 13 for the cottage cheese sample set and lab 8 and 10 for the deli turkey sample set may need more detail. In all cases the data set suggests that these labs were simply excluded since they detected false negative results which I am sure is not the case; however, no detail scientific explanation is provided. • While many of the acronyms used in the data and statistical tables are well known and are described in the Appendix J of the AOAC method validation guidelines, it may be beneficial to include a section on the explanation of these terms. Since this is my first time reviewing such a manuscript, I am not sure if these were included in the past but they may help some understand the tables.
ER 6	There are some mistakes in the report especially regarding the total number of positive samples for the 2 methods and that creates some controversy between text and results. For cottage cheese, page 13, from line 27, candidate method is claimed at 65/132 presumptive positive for low level when 66 shall be accounted (64 confirmed & 2 false positive, according to table 3). The report claimed 63 were confirmed when 64 were confirmed from the presumptive positive (from table 3) + 1 false negative (lab 8).The same applies to the reference method for which it's reported 73/132 positive for low level, but table 3 gave only 67 (the same number as for the MDA Listeria spp study which was using the same samples and therefore the same reference method and so results). These numbers will modify all the calculations reported in table 2014.1A. For deli turkey, page 14, from line 16, the false positive and false negative results obtained at low level for the candidate method shall be clearly disclosed: 66 presumptive positive results of which 64 were confirmed and 2 were not confirmed and so were false positive results. Additionally, 3 other samples were found negative by MDA Listeria monocytogenes but confirmed positive (3 false negative: one from lab 12 and 2 from lab 15). Did lab 12 participate in the ring trial (it has a note "b" in table 4 stipulating that results were not used ... If so the text shall be corrected accordingly; page 20 from line 8). Table 2014.2A, page 26, for lab 1, X = 5 for CP column: from table 3 page 19, I conclude that X = 6 as table 3 reports 5 MDA confirmed positive and 1 false positive (noted "c" & footnote claims false positive results). Same remark applies to lab 3 for which X = 7 when it shall be 8.
ER 7	NO

ER 8	None
Supporting Data and Information: Does data from collaborative study support the method as written?	
ER 1	Yes, except that Table A should be revised as described above.
ER 2	Yes.
ER 3	Yes
ER 4	Yes
ER 5	Since the cottage cheese is a posturized product, a heat stress inoculum would have been more appropriate; however, this was also not done during the pre-collaborative study. The deli turkey appears to have been stressed to an acceptable level.
ER 6	Yes & No. Yes when it relates to the DF-1 step enrichment protocol. No because DF-2 step enrichment protocols have not been evaluated during this collaborative study. That is my understanding that each of the different enrichment protocols must be evaluated during a collaborative study to be submitted to OMA first action: am I mistaken? Some of the claimed matrices require a 2-step enrichment protocol (bagged raw spinach and whole cantaloupe)
ER 7	yes
ER 8	Yes
Supporting Data and information: Does data collected support the criteria given in the collaborative study protocol?	
ER 1	Yes
ER 2	Yes.
ER 3	Yes
ER 4	yes
ER 5	YES, the data has been generated in accordance to the collaborative study protocol.
ER 6	The data supports the criteria regarding the use of DF broth and one step enrichment protocol.
ER 7	yes
ER 8	Yes
Are there any concerns regarding the safety of the method?	
ER 1	No.
ER 2	No.
ER 3	No
ER 4	No
ER 5	No
ER 6	To be covered once Safety Advisor review is presented.
ER 7	No
ER 8	No
Are there any concerns regarding the data manipulation, data tables, or statistical analysis?	
ER 1	N/A.
ER 2	No.
ER 3	No
ER 4	No
ER 5	I would like to bring the following points for discussion by the group: • While the AOAC

	guidelines under Appendix J, Annex F prescribe the analysis of the data set as a whole for the POD analysis, is there a need to also consider the equivalency of the methods in each individual lab. This may be outside of the realm of the guidelines, but I believe that this type of evaluation could be useful. For example in this method for the cottage chese, overall the combined data set produced 66 and 64 positive results for the presumptive and confirmed candidate results respectively, while a total of 73 positives were detected using the reference method. As a whole in this case the LCL and the UCL of the dPOD encompasses the 0 value showing no statistical differences. This is however not true for two of the participating laboratories, laboratory 7 and 9 both produced differences between the candidate and the reference method which are statistically significant. In both cases the candidate method produced results that were much lower than the reference method. Does this indicate that there was a particular problem with these sample sets, the method or the analysis? The table below shows an example of the results in lab 7 where the candidate method detected 4 confirmed positives whereas the reference method detected 10.The deli turkey sample set does not appear to have the same issue above. • It is interesting to see that sample 9 in the low sample set for cottage cheese of the candidate method for labs 7-15 all produced negative results and overall only one lab that in the included sample set produced a positive sample. To illustrate this point, 1 out of 11 labs had a positive sample giving a POD of 9%. This is much lower than the overall POD of about 50% for the sample set and falls outside of the statistical (normal) distribution expected with a 50 % fractional positive result. All other sample sets exhibit a more normal distribution of fractional positive results as would be expected. The same pattern does not repeat anywhere in the reference method data set. • The APC counts for the sample sets seem to have a broad range of values where the range covers 3 logs for cottage cheese and 4 logs for the deli turkey of counts. I was not expecting such a difference given the method that is used to enumerate these results is a well validated and used quantitative method.
ER 6	Some of the numbers need to be checked and edited accordingly.
ER 7	No
ER 8	No
General Comments (2)	
ER 1	None.
ER 2	Suggestions are given below for shortening the manuscript
ER 3	NA
ER 4	No additional comments
ER 5	
ER 6	
ER 7	
ER 8	
EDITORIAL EVALUATION CRITERIA Is the Validation Study Manuscript in a format acceptable to AOAC?	
ER 1	Yes
ER 2	Yes

ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Is the method described in sufficient detail so that it is relatively easy to understand, including equations and procedures for calculation of results (are all terms explained)?	
ER 1	Yes
ER 2	
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes

Are the figures and tables sufficiently explanatory without the need to refer to the text?	
ER 1	Yes
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	No
ER 6	Yes
ER 7	Yes
ER 8	Yes
Are all the figures and tables pertinent?	
ER 1	Yes
ER 2	No
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
Could some be omitted and covered by a simple statement?	
ER 1	No
ER 2	Yes
ER 3	No
ER 4	No
ER 5	
ER 6	No
ER 7	No
ER 8	Yes
Are the references complete and correctly annotated?	
ER 1	Yes
ER 2	No
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes

Does the method contain adequate safety precaution reference and/or statements?	
ER 1	No
ER 2	Yes
ER 3	Yes
ER 4	Yes
ER 5	Yes
ER 6	Yes
ER 7	Yes
ER 8	Yes
General Comments (3)	
ER 1	There should be a reminder in the cautionary statement that this test may generate high levels of L. monocytogenes, which can particularly dangerous to the immunocompromised and pregnant women.
ER 2	Page 1, lines 27-28: add "using heat stressed cells" to this sentence. Page 1, lines 28-29: add "using non-stressed cells" to this sentence. Page 2, line 24: change "compare the" to " compare the reproducibility of the". Page 2, line 25: change "to the" to "to the reproducibilities of the methods of the". [CONTINUED] Page 2, line 38: change "with Listeria" to "with heat stressed Listeria". Page 4, line 16: change "(12 high, 12 low, and 12 controls for each method)" to "(12 high inoculum, 12 low inoculum, and 12 uninoculated controls for each method)". Page 7, line 29 to page 12 line 32: Omit this material. Refer to the 3M MDA Listeria spp. manuscript for the relevant material taking care to emphasize the use of the L. monocytogenes reagent already listed in section B(b). Page 13, lines 12 and 14: Omit reference to Appendix. Page 16, references 5, 6, 8: These will not necessarily be accessible to the manuscript readers even if the website were properly provided. The corresponding J. AOAC Int. manuscripts might be more readily available. Page 18, Table 2: Add a footnote detailing the stress temperature and time. Page 21: Omit this blank page. Pages 22 and 23, Tables 2014.1A and .1B: These would be clearer if blank lines separated the five subsections. Also, in both Tables the Candidate Confirmed Positive (CC) sections could well be omitted and the results described in the narrative. It is somewhat confusing that they are not already mentioned in the narrative as are CP and C section results. Page 24, Appendix: Omit this page. Pages 32-37, Fig. titles and Figs: Omit figures
ER 3	NA
ER 4	No additional comments
ER 5	
ER 6	Few more edits to be covered. Will be discussed during the meeting.
ER 7	
ER 8	

RECOMMENDATION: **Do you recommend that the ERP adopt this method as an AOAC *Official Methods of Analysis (First Action status)*?**	
ER 1	Yes.
ER 2	Yes.
ER 3	Yes
ER 4	Yes
ER 5	YES, while some issues may require discussion, the method has proven its performance.
ER 6	Yes but limited to DF and the one step enrichment protocol. Therefore, matrix claim shall be narrowed. To be discussed during the ERP meeting.
ER 7	Yes
ER 8	Yes
AFTER FIRST ACTION STATUS: **Is there any additional information that the ERP should consider in order to recommend the method for Final Action status?**	
ER 1	Two years of use in the greater food micro community.
ER 2	No.
ER 3	NA
ER 4	No additional comments
ER 5	NA
ER 6	Recommendation to monitor the false positive rates generated by the method.
ER 7	No
ER 8	User comments/concerns

AOAC Official Method 2014.08
Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood

Gas Chromatography-Mass Spectrometry
First Action 2014

[Applicable for the determination of the following PAHs in mussel, oyster, and shrimp: 1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene. These were representative PAH analytes selected for the collaborative study. The method has been single-laboratory validated for 32 PAHs in fish and shrimp (1), and, therefore, is expected to be applicable to other GC-amenable PAHs and seafood matrices. The concentration ranges evaluated within the collaborative study are given in Table **2014.08A**.]

Caution: *See Appendix B: Laboratory Safety*. Use appropriate personal protective equipment such as laboratory coat, safety glasses or goggles, appropriate chemical-resistant gloves, and a fume hood. Dispose of solvents and solutions according to federal, state, and local regulations. Always handle open containers of solvents inside the fume hood, including the pouring, mixing, evaporating, and preparing standard solution. Keep containers covered or closed when not in use.

Hexane and isooctane.—Highly flammable, liquid irritants. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation.

Ethyl acetate.—Highly flammable, liquid irritants. Harmful if swallowed in quantity. Vapors may cause drowsiness.

Toluene.—Highly flammable, liquid irritant. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation. May cause drowsiness. Possible teratogen.

Dichloromethane.—Noncombustible, liquid irritant. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation. Asphyxiant. Causes central nervous system (CNS) depression. Possible carcinogen and mutagen.

PAHs.—Carcinogens, respiratory sensitizers, teratogens, reproductive hazard, mutagens. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation.

See Tables **2014.08B–D** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Homogenized seafood samples (10 g sample with a 5 µg/kg addition of ^{13}C-PAH surrogate mixture) are mixed with 5 mL water (or 10 mL water in the case of shrimp and other more viscous samples) and shaken vigorously by hand with 10 mL ethyl acetate in a 50 mL polypropylene centrifuge tube for 1 min. Subsequently, 4 g anhydrous magnesium sulfate and 2 g sodium chloride are added to the mixture to induce phase separation and force the analytes into the ethyl acetate layer. The tube is again shaken by hand for 1 min and then centrifuged for 10 min at >1500 rcf. A 5 mL aliquot of the ethyl acetate layer is evaporated, reconstituted in 1 mL hexane, and cleaned on an SPE column with 1 g silica gel and approximately 0.2 g anhydrous sodium sulfate on the top. The column is conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane, followed by application of the 1 mL extract in hexane. The analytes are eluted with hexane–dichloromethane (3 + 1, v/v) using volume determined for the given silica gel SPE cartridges from the elution profiles of target analytes and fat, which are dependent on the silica deactivation. The clean extract is carefully evaporated, reconstituted in 0.5 mL isooctane, and analyzed by GC/MS. *See* Figure **2014.08A** for the method flow chart.

B. Apparatus

(**a**) *Homogenizer*.—WARING blender Model 38BL40 (Conair Corp., Stamford, CT) or equivalent.

(**b**) *Solvent evaporator*.—Any suitable solvent evaporator, such as a rotary vacuum evaporator, Kuderna-Danish evaporator,

Table 2014.08A. PAH and ^{13}C-PAH concentrations in the calibration standard solutions

	Concentration, µg/L				Equivalent concentration, µg/kg			
Calibration level	BaP and others[a]	Chr and others[b]	Naph[c]	^{13}C-PAHs	BaP and others	Chr and others	Naph	^{13}C-PAHs
1	5	12.5	25	50	0.5	1.25	2.5	5
2	10	25	50	50	1	2.5	5	5
3	20	50	100	50	2	5	10	5
4	50	125	250	50	5	12.5	25	5
5	100	250	500	50	10	25	50	5
6	200	500	1000	50	20	50	100	5
7	500	1250	2500	50	50	125	250	5
8	1000	2500	5000	50	100	250	500	5

[a] Analytes at 10 µg/mL in the mixed stock standard solution.

[b] Analytes at 25 µg/mL in the mixed stock standard solution.

[c] Analytes at 50 µg/mL in the mixed stock standard solution.

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Table 2014.08B. Statistical results for the studied PAHs at three different concentration levels in shrimp after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	9	18	21.7	108.6	2.6	4.1	11.8	18.9	0.66
	9	18	22.7	113.7	1.8	4.2	8.0	18.7	0.66
	9	18	21.7	108.3	1.9	4.4	8.8	20.4	0.72
1-MN	9	18	23.1	115.4	6.2	6.8	26.9	29.4	1.04
	9	18	81.5	108.6	6.7	15.6	8.3	19.1	0.82
	9	18	203.2	101.6	17.8	55.4	8.8	27.3	1.34
1-MP	9	18	10.0	99.9	1.0	1.4	9.8	14.0	0.44
	9	18	25.0	99.9	1.9	3.7	7.6	14.9	0.53
	9	18	119.4	95.5	6.7	15.7	5.6	13.1	0.60
2,6-DMN	8	16	15.6	103.9	1.1	2.6	6.9	16.4	0.55
	8	16	37.8	94.6	4.8	7.4	12.6	19.5	0.74
	7	14	146.7	83.8	16.6	20.3	11.3	13.9	0.65
6-MC	9	18	11.1	110.5	0.6	1.5	5.8	13.1	0.42
	9	18	32.2	107.2	1.6	3.6	5.1	11.1	0.42
	9	18	145.2	100.1	8.7	13.7	6.0	9.4	0.44
Ant	9	18	4.9	98.5	0.3	0.5	6.7	10.3	0.29
	9	18	10.6	105.7	0.8	1.7	7.3	16.2	0.51
	9	18	38.9	97.4	2.4	4.6	6.2	11.7	0.45
BaA	9	18	4.8	95.9	0.3	0.5	7.0	9.7	0.27
	9	18	15.0	99.9	0.6	1.3	4.3	8.4	0.28
	9	18	56.6	94.4	2.5	5.2	4.5	9.2	0.37
BaP	9	18	1.9	96.2	0.1	0.2	6.5	12.1	0.29
	9	18	4.9	98.7	0.4	0.5	7.3	9.6	0.27
	8	16	23.1	92.3	1.1	1.6	4.6	7.0	0.25
BbF	9	18	4.8	96.7	0.3	0.5	6.6	10.1	0.28
	9	18	9.8	98.2	0.3	0.7	2.6	7.0	0.22
	9	18	71.6	95.5	3.9	6.3	5.5	8.8	0.37
BghiP	8	16	1.9	94.7	0.1	0.2	7.0	11.7	0.28
	8	16	4.9	98.5	0.2	0.5	4.3	9.9	0.28
	8	16	18.0	90.1	1.0	1.4	5.7	7.9	0.27
BkF	9	18	2.0	99.5	0.1	0.3	6.2	13.7	0.34
	9	18	8.1	101.7	0.4	0.7	4.9	8.7	0.26
	9	18	38.3	95.8	1.8	2.8	4.7	7.2	0.28
Chr	8	16	15.2	101.5	0.5	1.4	3.1	9.4	0.31
	9	18	50.7	101.4	2.2	4.1	4.4	8.1	0.32
	9	18	167.4	95.6	9.0	14.5	5.3	8.7	0.41
DBahA	9	18	1.9	95.9	0.2	0.3	10.9	13.5	0.33
	9	18	5.0	100.4	0.3	0.6	6.6	11.2	0.32
	9	18	13.8	91.8	0.9	1.2	6.4	8.4	0.28
Fln	8	16	5.2	103.0	0.1	0.5	1.4	10.0	0.28
	9	18	15.4	102.3	0.6	1.1	4.2	7.5	0.25
	9	18	47.3	94.7	2.1	4.1	4.5	8.7	0.34
Flt	9	18	9.7	97.2	0.6	1.0	6.0	10.4	0.32
	9	18	25.1	100.3	1.4	2.4	5.5	9.7	0.35
	9	18	93.9	93.9	4.9	8.7	5.2	9.3	0.41
IcdP	9	18	2.0	98.2	0.1	0.3	5.3	13.3	0.32
	9	18	5.1	102.2	0.5	0.6	9.1	11.0	0.31

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Table 2014.08B. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	9	18	18.4	92.1	1.1	1.9	5.7	10.5	0.36
Naph	8	16	27.7	110.7	2.8	2.8	10.3	10.3	0.37
	9	18	84.1	105.1	5.6	8.8	6.7	10.5	0.45
	8	16	158.7	99.2	9.7	34.2	6.1	21.6	1.02
Phe	8	16	15.1	100.5	0.5	1.2	3.3	7.8	0.26
	9	18	49.7	99.4	1.5	3.0	3.1	6.0	0.24
	9	18	168.0	96.0	8.6	16.6	5.1	9.9	0.47
Pyr	9	18	14.8	98.5	0.9	1.3	6.1	8.8	0.29
	9	18	40.3	100.8	1.6	3.3	3.8	8.2	0.32
	8	16	118.7	95.0	2.9	6.4	2.5	5.4	0.25

or a nitrogen blow-down system, may be used as long as it provides results meeting the laboratory qualification/method set-up requirements (absolute analyte recoveries >70% in both evaporation steps).

(**c**) *Centrifuge*.—Capable of centrifugation of 50 mL tubes at >1500 rcf for 10 min.

(**d**) *Furnace/oven*.—Capable of 600°C operation.

(**e**) *Balance(s)*.—Analytical, capable of accurately measuring weights from 1 mg to 10 g.

(**f**) *Gas chromatograph-mass spectrometer*.—Any GC/MS instrument [single quadrupole, triple quadrupole, time-of-flight (TOF), or ion trap] with electron ionization (EI) may be used as long as it provides results meeting the laboratory qualification requirements (to provide reliable results for the calibration range specified in Table **2014.08A**).

(**g**) *GC column*.—Capillary column BPX-50 (30 m, 0.25 mm id, 250 µm film thickness; Trajan Scientific, Austin, TX, USA) or equivalent (USP specification G3), such as Rxi-17Sil MS (Restek Corp., Bellefonte, PA, USA); DB-17MS, DB-17, or HP-50 (Agilent Technologies, Santa Clara, CA, USA); or any other column that enables adequate separation of PAHs as specified in the laboratory qualification requirements (*see* **G**).

C. Reagents and Materials

(**a**) *Hexane.*—>98.5%, mixture of isomers.

(**b**) *Isooctane.*—ACS or better grade.

(**c**) *Ethyl acetate.*—>99.5%, for GC residue analysis.

(**d**) *Dichloromethane.*—≥99.9%, for GC residue analysis.

(**e**) *Toluene.*—≥99.9%, for GC residue analysis.

(**f**) *Water.*—Purified, free of interfering compounds.

(**g**) *Anhydrous sodium sulfate (Na_2SO_4).*—≥99.0%, powder, heated at 600°C for 7 h and then stored in a desiccator before use (Na_2SO_4 prepared and stored as indicated can be used for 1 month from preparation).

(**h**) *Silica gel SPE column.*—Containing 1 g silica gel. Any commercially available silica gel SPE column can be used as long as it provides adequate fat cleanup and meets requirements for low background contamination specified in the laboratory qualification requirements: the concentrations of all analytes in the reagent blanks had to be below the concentrations in the lowest calibration level standard; for naphthalene, levels below the second lowest calibration standard (equivalent to 5 ng/g naphthalene in the sample) are still acceptable if the source of contamination could not be eliminated.

Silica gel SPE columns can be prepared in-house using the following procedure: Activate the silica gel by heating at 180°C for 5 h, and then deactivate it by adding 5% deionized water, shaking for 3 h. Store in a desiccator for 16 h before use (silica gel prepared and stored as indicated can be used for 14 days). Place a piece of deactivated glass wool in a Pasteur pipet (5 mL), add 1 g activated silica gel (Silica gel 60, 0.063–0.2 mm, 70–230 mesh or equivalent) and top it with approximately 0.2 g muffled anhydrous Na_2SO_4.

(**i**) *Anhydrous magnesium sulfate ($MgSO_4$).*—≥99.0%, powder, heated (muffled) at 600°C for 7 h, and then store in a desiccator before use ($MgSO_4$ prepared and stored as indicated can be used for 1 month from preparation). *Note*: A preweighed (commercially available) mixture of 2 g sodium chloride and 4 g anhydrous magnesium sulfate (muffled) in pouches or tubes can be used.

(**j**) *Sodium chloride (NaCl).*—≥99.0%.

(**k**) *Helium 5.0 or better, nitrogen 4.0 or better.*

(**l**) *Polypropylene centrifuge tubes.*—50 mL.

(**m**) *Glass Pasteur pipet.*—5 mL (for solvent transfers and/or in-house preparation of silica gel minicolumns).

(**n**) *Syringes/pipets.*—Capable of accurate measurement and transfer of appropriate volumes for standard solution preparation and sample fortification (50–1000 µL).

(**o**) *Volumetric flasks.*—5–100 mL.

(**p**) *Glassware for evaporation steps*.—Depending on the evaporation technique (e.g., small round-bottom flasks, suitable tubes, or glassware for Kuderna-Danish evaporation). It is recommended to heat the glassware for at least 2 h at 250°C to remove potential contamination.

D. Reference Standards

(**a**) *PAH standards.*—High-purity reference standards of the PAH analytes (1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene).

(**b**) *U.S. Environmental Protection Agency (EPA) 16 PAH cocktail.*—(^{13}C, 99%), Product No. ES-4087 (5 µg/mL, 1.2 mL in nonane), Cambridge Isotope Labs (Tewksbury, MA, USA) or equivalent.

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Table 2014.08C. Statistical results for the studied PAHs at three different concentration levels in mussel after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	10	20	38.1	95.3	5.7	8.8	14.9	23.2	0.89
	9	18	39.4	98.6	4.6	8.3	11.7	21.1	0.81
	10	20	38.9	97.3	4.6	8.9	11.9	22.8	0.87
1-MN	10	20	19.3	96.5	3.1	4.5	16.2	23.2	0.80
	10	20	96.9	96.9	9.0	19.2	9.2	19.8	0.87
	8	16	208.7	104.4	26.6	26.6	12.7	12.7	0.63
1-MP	9	18	9.8	98.1	0.7	1.8	7.2	18.0	0.56
	9	18	45.8	91.6	5.3	9.6	11.6	21.0	0.82
	9	18	117.2	93.7	10.2	24.4	8.7	20.8	0.94
2,6-DMN	10	20	13.8	91.9	1.5	2.9	11.2	20.7	0.68
	8	16	65.4	87.2	3.1	13.6	4.8	20.7	0.86
	10	20	153.8	87.9	10.7	36.8	6.9	23.9	1.13
3-MC	10	20	9.9	98.6	0.7	1.7	7.6	16.7	0.52
	10	20	87.2	96.8	4.1	10.9	4.7	12.5	0.54
	10	20	138.0	95.2	3.8	16.7	2.8	12.1	0.56
Ant	10	20	3.9	77.9	0.3	1.3	6.7	32.5	0.88
	9	18	13.0	86.8	1.7	3.2	12.8	24.8	0.81
	9	18	31.5	78.8	1.4	7.9	4.6	25.0	0.93
BaA	10	20	4.3	85.1	0.3	0.7	6.9	15.9	0.44
	10	20	21.5	85.9	1.1	2.3	5.1	10.9	0.38
	8	16	52.6	87.7	1.3	2.2	2.5	4.2	0.17
BaP	9	18	1.6	79.2	0.1	0.3	5.6	20.5	0.49
	9	18	8.1	80.5	0.6	1.4	6.9	17.7	0.54
	9	18	19.3	77.3	0.6	2.6	3.0	13.4	0.46
BbF	10	20	4.7	94.4	0.3	0.5	7.2	10.6	0.30
	10	20	27.1	90.2	1.9	2.8	6.9	10.2	0.37
	10	20	69.1	92.1	2.1	5.8	3.0	8.4	0.35
BghiP	9	18	2.0	98.1	0.1	0.2	4.9	10.6	0.26
	10	20	9.3	92.7	0.4	1.1	4.4	12.1	0.37
	10	20	17.9	89.6	0.6	1.5	3.2	8.6	0.29
BkF	9	18	1.9	97.4	0.1	0.2	7.2	10.3	0.25
	10	20	18.5	92.7	1.1	2.4	6.2	12.8	0.44
	10	20	36.6	91.5	1.0	3.8	2.7	10.4	0.40
Chr	10	20	14.2	94.4	0.8	1.3	5.7	9.5	0.31
	10	20	91.6	91.6	4.1	8.6	4.5	9.4	0.41
	10	20	159.8	91.3	4.9	11.8	3.1	7.4	0.35
DBahA	10	20	1.9	93.1	0.2	0.2	9.1	11.2	0.27
	10	20	9.1	90.5	0.5	1.2	5.4	13.5	0.41
	10	20	13.6	90.8	0.5	1.2	4.0	8.5	0.28
Fln	10	20	5.4	107.7	0.2	0.6	3.5	10.6	0.30
	10	20	25.5	101.8	1.0	1.9	3.7	7.6	0.27
	9	18	48.1	96.2	1.7	2.4	3.5	4.9	0.20
Flt	9	18	10.2	102.4	1.1	1.4	10.7	13.2	0.41
	10	20	48.9	97.7	2.7	4.3	5.5	8.8	0.35
	9	18	93.3	93.3	4.9	6.6	5.3	7.1	0.31
IcdP	10	20	2.0	97.7	0.1	0.2	7.3	11.3	0.28
	10	20	9.4	93.7	0.5	1.1	5.6	11.9	0.37

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Table 2014.08C. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	10	20	17.9	89.3	0.9	1.6	4.9	8.8	0.30
Naph	9	18	23.7	94.6	1.9	4.0	8.1	17.1	0.61
	10	20	105.9	84.7	10.1	26.7	9.5	25.2	1.12
	8	16	146.7	91.7	7.2	19.2	4.9	13.1	0.61
Phe	8	16	14.5	96.8	0.8	0.9	5.3	6.1	0.20
	8	16	93.1	93.1	3.9	8.5	4.1	9.2	0.40
	8	16	160.8	91.9	5.8	13.0	3.6	8.1	0.38
Pyr	10	20	14.2	94.6	0.7	1.3	5.0	9.3	0.31
	10	20	71.5	95.4	2.9	7.0	4.0	9.8	0.41
	10	20	116.5	93.2	4.5	9.6	3.8	8.3	0.37

Containing: Acenaphthene ($^{13}C_6$, 99%), acenaphthylene ($^{13}C_6$, 99%), anthracene ($^{13}C_6$, 99%), benz[*a*]anthracene ($^{13}C_6$, 99%), benzo[*b*]fluoranthene ($^{13}C_6$, 99%), benzo[*k*]fluoranthene ($^{13}C_6$, 99%), benzo[*g,h,i*]perylene ($^{13}C_{12}$, 99%), benzo[*a*]pyrene ($^{13}C_4$, 99%), chrysene ($^{13}C_6$, 99%), dibenz[*a,h*]anthracene ($^{13}C_6$, 99%), fluoranthene ($^{13}C_6$, 99%), fluorene ($^{13}C_6$, 99%), indeno[1,2,3-*cd*]pyrene ($^{13}C_6$, 99%), naphthalene ($^{13}C_6$, 99%), phenanthrene ($^{13}C_6$, 99%), and pyrene ($^{13}C_6$, 99%).

E. Preparation of Standard Solutions

(**a**) *Individual stock solutions.*—Prepare individual PAH stock solutions at approximately 1000 or 2500 µg/mL in toluene.

(**b**) *Mixed stock standard solution.*—Use analyte individual stock solutions to obtain a mixed solution of each PAH at 10 µg/mL (for benzo[*a*]pyrene and other low-level PAHs) or 25 µg/mL (for chrysene and other higher-level PAHs) or 50 µg/mL (for naphthalene) in isooctane. *See* Table **2014.08E** for analyte concentrations in the mixed stock standard solution.

(**c**) *Working PAH Solution A.*—Accurately transfer 0.5 mL of the mixed stock standard solution into a 5 mL volumetric flask and dilute to volume with isooctane.

(**d**) *Working PAH Solution B.*—Accurately transfer 0.5 mL of the Working PAH Solution A into a 5 mL volumetric flask and dilute to volume with isooctane.

(**e**) *Internal standard solution.*—Prepare 1 µg/mL solution of ^{13}C-PAHs in isooctane by 5-fold dilution of the 5 µg/mL EPA 16 ^{13}C-PAHs cocktail with isooctane.

(**f**) *Calibration standard solutions.*—Prepare eight levels of calibration standard solutions (1 mL each) in 2 mL amber screw-cap vials. It is recommended to distribute small portions (enough for a single injection) of the calibration standard solutions into multiple crimp-top vials with 100 µL deactivated glass inserts. *See* Table **2014.08A** for analyte concentrations in the calibration standards and Table **2014.08F** for the dilution scheme.

(*1*) *For level 1 calibration standard.*—Accurately transfer 50 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 900 µL isooctane. Cap the vial and vortex mix briefly.

(*2*) *For level 2 calibration standard.*—Accurately transfer 100 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

(*3*) *For level 3 calibration standard.*—Accurately transfer 200 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 750 µL isooctane. Cap the vial and vortex mix briefly.

(*4*) *For level 4 calibration standard.*—Accurately transfer 500 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 450 µL isooctane. Cap the vial and vortex mix briefly.

(*5*) *For level 5 calibration standard.*—Accurately transfer 100 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

(*6*) *For level 6 calibration standard.*—Accurately transfer 200 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 750 µL isooctane. Cap the vial and vortex mix briefly.

(*7*) *For level 7 calibration standard.*—Accurately transfer 500 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 450 µL isooctane. Cap the vial and vortex mix briefly.

(*8*) *For level 8 calibration standard.*—Accurately transfer 100 µL of the mixed stock standard solution into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

F. Extraction and Cleanup Procedure

(*1*) Add 50 µL of the 1 µg/mL ^{13}C-PAHs solution to 10 ± 0.1 g of thoroughly homogenized seafood sample in a 50 mL polypropylene centrifuge tube.

(*2*) Vortex sample for 15 s and let equilibrate for 15 min.

(*3*) Add 5 mL (10 mL in the case of shrimp) of purified water and 10 mL ethyl acetate.

(*4*) Shake tube vigorously by hand for 1 min.

(*5*) Add 4 g of muffled anhydrous magnesium sulfate and 2 g sodium chloride, and seal the tube well (ensure that powder does not get into the screw threads or rim of the tube).

(*6*) Shake tube vigorously by hand for 1 min, ensuring that crystalline agglomerates are broken up sufficiently during shaking.

(*7*) Centrifuge tube at >1500 rcf for 10 min.

(*8*) Take a 5 mL aliquot of the upper ethyl acetate layer, add 50 µL isooctane as a keeper, and gently evaporate all ethyl acetate until only isooctane and co-extracted sample fat are left.

(*9*) Reconstitute in 1 mL hexane.

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Table 2014.08D. Statistical results for the studied PAHs at three different concentration levels in oyster after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	8	16	72.3	90.4	4.1	11.7	5.7	16.2	0.68
	8	16	69.0	86.2	4.9	12.6	7.1	18.3	0.76
	8	16	65.6	82.0	5.5	13.2	8.4	20.1	0.83
1-MN	8	16	67.9	90.5	4.3	14.8	6.4	21.8	0.91
	9	18	90.8	90.8	20.6	28.9	22.7	31.9	1.39
	9	18	236.6	94.6	26.4	55.3	11.2	23.4	1.18
1-MP	7	14	20.2	80.8	1.8	4.8	8.8	23.9	0.83
	8	16	39.9	79.8	2.6	7.7	6.4	19.3	0.74
	8	16	154.7	77.3	16.9	34.7	10.9	22.4	1.06
2,6-DMN	7	14	30.5	76.2	3.0	5.3	9.8	17.5	0.65
	7	14	57.9	77.2	4.9	10.8	8.5	18.6	0.76
	7	14	161.3	71.7	12.7	20.9	7.9	13.0	0.62
3-MC	9	18	27.8	92.5	1.7	3.6	6.0	13.0	0.47
	9	18	79.8	88.6	5.1	10.3	6.4	13.0	0.55
	9	18	196.8	87.5	10.1	23.5	5.1	12.0	0.59
Ant	7	14	5.3	53.2	0.5	2.9	8.8	55.0	1.56
	7	14	7.5	50.3	0.8	4.9	10.0	64.7	1.94
	6	12	34.0	56.6	3.0	15.1	8.8	44.5	1.67
BaA	9	18	10.9	72.6	0.8	2.2	7.7	19.7	0.62
	9	18	17.2	68.6	1.0	3.6	5.8	21.1	0.71
	9	18	71.6	71.6	3.9	12.5	5.4	17.5	0.73
BaP	9	18	2.5	49.7	0.3	1.1	11.9	43.5	1.10
	9	18	4.8	48.2	0.4	2.0	9.0	42.2	1.18
	9	18	24.6	49.3	1.6	10.0	6.4	40.5	1.45
BbF	9	18	8.6	85.9	0.6	1.0	6.6	11.6	0.35
	9	18	24.6	81.8	1.7	2.7	7.1	11.2	0.40
	9	18	82.8	82.8	3.4	9.8	4.1	11.9	0.51
BghiP	9	18	4.1	82.4	0.2	0.5	5.9	12.3	0.34
	9	18	8.2	81.9	0.7	1.1	8.6	13.6	0.41
	9	18	19.6	78.4	1.0	2.3	4.9	11.7	0.41
BkF	9	18	6.9	85.9	0.5	1.1	7.7	16.3	0.48
	9	18	16.9	84.3	1.1	2.6	6.5	15.4	0.52
	9	18	62.9	83.8	3.8	8.2	6.1	13.1	0.54
Chr	9	18	43.0	85.9	2.8	4.3	6.5	9.9	0.39
	9	18	81.6	81.6	5.0	8.6	6.2	10.6	0.45
	9	18	204.1	81.6	8.7	19.0	4.3	9.3	0.46
DBahA	9	18	4.1	82.7	0.4	0.5	9.0	13.0	0.35
	8	16	8.2	82.2	0.6	1.1	7.5	13.4	0.41
	9	18	16.0	80.0	0.7	2.0	4.4	12.7	0.43
Fln	9	18	12.5	83.3	1.0	1.9	8.2	15.4	0.50
	9	18	20.3	81.2	1.4	3.0	6.8	14.6	0.51
	9	18	57.0	76.0	2.3	11.3	4.0	19.9	0.81
Flt	9	18	22.0	88.2	2.1	3.6	9.5	16.2	0.57
	9	18	42.0	83.9	5.1	7.5	12.2	17.9	0.69
	9	18	120.7	80.5	7.0	17.1	5.8	14.2	0.65
IcdP	9	18	4.3	86.8	0.4	0.6	9.6	13.1	0.36
	9	18	8.3	83.3	0.8	1.1	9.0	13.7	0.42

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Table 2014.08D. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	9	18	20.0	80.1	1.0	2.2	4.8	10.7	0.37
Naph	9	18	71.0	88.7	5.3	9.4	7.5	13.2	0.55
	9	18	106.2	84.9	7.3	14.7	6.9	13.9	0.62
	8	16	193.9	86.2	6.0	29.8	3.1	15.4	0.75
Phe	9	18	41.6	83.2	3.0	5.5	7.2	13.2	0.51
	9	18	80.3	80.3	6.1	10.7	7.6	13.3	0.57
	8	16	203.9	81.6	9.5	22.5	4.7	11.0	0.54
Pyr	9	18	34.0	85.1	2.2	3.3	6.4	9.8	0.37
	8	16	63.2	84.3	2.2	5.3	3.5	8.4	0.35
	9	18	163.4	81.7	8.0	16.6	4.9	10.2	0.48

(*10*) Condition a silica SPE column (1 g silica gel with approximately 0.2 g of muffled anhydrous sodium sulfate on the top) with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane.

(*11*) Apply the extract in hexane onto the silica SPE cartridge.

(*12*) Elute with hexane–dichloromethane (3 + 1, v/v) using volume determined for the given silica gel SPE cartridges from the elution profiles of target analytes and fat, which are dependent on the silica deactivation, *see Note* (*4*) below. Collect the eluent.

(*13*) Add 0.5 mL isooctane (and 1–2 mL ethyl acetate) to the eluent as a keeper and gently evaporate down to 0.5 mL to remove hexane and dichloromethane from the final extract.

(*14*) Transfer the final extract into an autosampler vial for the GC/MS analysis.

Notes: (*1*) The fat capacity of the 1 g silica gel SPE column is approximately 0.1 g. If the 5 mL ethyl acetate extract aliquot contains more than 0.1 g fat, it is necessary to use a smaller aliquot volume to avoid sample breakthrough during the cleanup step.

(*2*) Ethyl acetate should not be present in the extract applied to the silica cartridge because it can affect the extract polarity, thus potentially retention of fat and analytes on the silica gel. The coextracted fat and 50 µL isooctane act as keepers during the first evaporation step (step *8*), thus the evaporation should be conducted gently until there is no significant change in the volume, i.e., until only the isooctane and coextracted fat are left in the evaporation tube or flask.

(*3*) Addition of 1–2 mL ethyl acetate to the eluent in step *13* is recommended for a better control of the evaporation process and higher absolute recoveries of volatile PAHs.

(*4*) The deactivation and storage of silica gel SPE cartridges can vary, potentially resulting in different amounts of water in the silica, thus its potentially different retention characteristics. Therefore, it is important to test the elution profiles of PAHs and fat and determine the optimum volume of the elution solvent to ensure adequate analyte recoveries and fat cleanup. The following procedure is recommended:

(**a**) Prepare a PAH solution in hexane by combining 50 µL of the Working PAH Solution A and 1 mL hexane in a vial. Mix well and apply onto a silica SPE column (1 g silica gel with approximately 0.2 g of muffled anhydrous sodium sulfate on the top), which was conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane.

(**b**) Elute with 10 mL hexane–dichloromethane (3 + 1, v/v), collecting 0.5 mL elution fractions in 20 evaporation tubes or flasks. Add 0.5 mL isooctane to each elution fraction and evaporate down to 0.5 mL using the optimized evaporation conditions. Analyze each fraction by GC/MS.

(**c**) Determine PAH elution profile by plotting analyte response (peak area or height) in a given fraction normalized to the sum of analyte responses in all tested fractions vs the elution volume. *See* Figure **2014.08B** for an example of a PAH elution profile. It is recommended to add an additional 0.5 mL on top of the determined elution fraction (corresponding to 100% recovery) as a safety margin ensuring good analyte recoveries in routine practice. This would result in the optimum elution volume of 7 mL for the silica cartridge tested in Figure **2014.08B**.

(**d**) To check the effectiveness of fat removal, dissolve 100 mg pure fish oil (or any suitable fat) in 1 mL hexane and apply it onto the silica gel cartridge, which was conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane. Elute with the optimum elution volume of hexane–dichloromethane (3 + 1, v/v), which was determined in the previous step (e.g., 7 mL for the example in Figure **2014.08B**). Collect this fraction in an evaporation tube or flask, which empty weight (after heating in an oven to remove moisture) was recorded to four decimal places

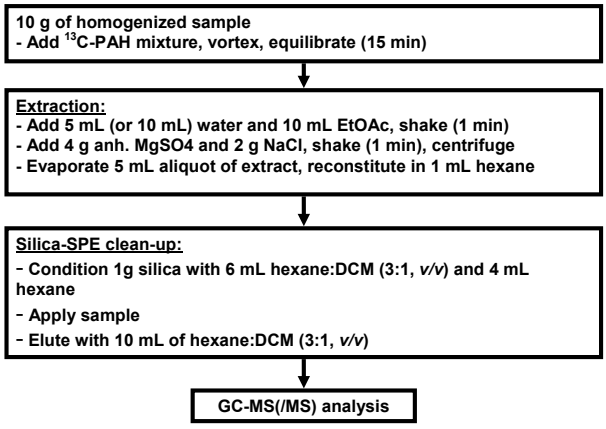

Figure 2014.08A. Flow chart of the method for determination of PAHs in seafood using GC/MS.

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Table 2014.08E. Analyte concentrations in the mixed stock standard solution

Analyte	Concentration, µg/mL
Anthracene	10
Benz[*a*]anthracene	10
Benzo[*a*]pyrene	10
Benzo[*b*]fluoranthene	10
Benzo[*g,h,i*]perylene	10
Benzo[*k*]fluoranthene	10
Chrysene	25
Dibenz[*a,h*]anthracene	10
Fluoranthene	25
Fluorene	10
Indeno[1,2,3-*cd*]pyrene	10
Naphthalene	50
Phenanthrene	25
Pyrene	25
1-Methylnaphthalene	25
2,6-Dimethylnaphthalene	25
1-Methylphenanthrene	25
1,7-Dimethylphenanthrene	10
3-Methylchrysene	25

using an analytical balance. Elute the cartridge with additional 3 × 1 mL hexane–dichloromethane (3 + 1, v/v) and collect in three evaporation tubes/flasks of known empty weight.

(**e**) Evaporate the four elution fractions to dryness and gravimetrically determine the amount of fat eluting in each fraction by subtracting the empty weights from newly recorded weights after solvent evaporation. There should be no fat eluting in the optimum elution fraction for PAHs (this can also be observed visually in the tubes).

(**f**) If there is fat coeluting with PAHs, then the PAH and fat elution profiles have to be reexamined to determine optimum elution volume for PAH and fat separation (potentially sacrificing up to 5% of late-eluting PAH amounts if necessary) or a different silica gel cartridge has to be used.

Table 2014.08F. Dilution scheme for preparation of the calibration standard solutions

Calibration level	Vol. of mixed stock standard solution, µL	Vol. of working PAH solution[a], µL	Vol. of working PAH solution B, µL	Vol. of ^{13}C-PAH 1 µg/mL solution, µL	Final vol.[a], µL
1	—	—	50	50	1000
2	—	—	100	50	1000
3	—	—	200	50	1000
4	—	—	500	50	1000
5	—	100	—	50	1000
6	—	200	—	50	1000
7	—	500	—	50	1000
8	100	—	—	50	1000

[a] Bring to volume using isooctane.

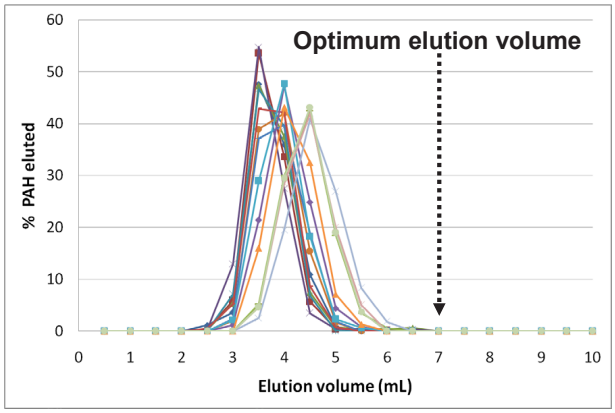

Figure 2014.08B. An example of elution profiles of PAHs on a silica gel SPE cartridge and determination of the optimum elution volume.

G. GC/MS Analysis

(**a**) *GC conditions.*—Table **2014.08G** provides GC conditions that were used by the collaborative study participants. Other conditions (e.g., column, temperature and flow program, and injection technique and volume) can be used as long as the laboratory qualification criteria for separation, sensitivity, and linearity are met. The injection temperature or program needs to be optimized to enable quantitative transfer of less volatile PAHs. If programmable temperature vaporizer (PTV) solvent vent mode is used, solvent venting parameters (temperature, time, flow, pressure) need to be carefully optimized to prevent losses of the volatile PAHs, especially naphthalene. The separation criteria (demonstrated in Figure **2014.08C**) include (*1*) a baseline separation of benzo[*a*]pyrene and benzo[*e*]pyrene (concentration ratio of 1:5), (*2*) at least 50% valley separation of anthracene and phenanthrene (concentration ratio 1:2.5; evaluated for the anthracene peak), and (*3*) at least 50% valley separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*]fluoranthene (concentration ratio of 1:1:1). *Note*: Criteria for separation of chrysene and triphenylene (another PAH critical pair) were not set for the collaborative study. For accurate quantitation of chrysene, at least 50% valley separation is recommended, which can be achieved using selective stationary phases.

The maximum oven temperature program may not exceed the maximum temperature limit for a given column. Backbone-modified columns, such as Rxi-17Sil MS or DB-17MS, are recommended for their better temperature stability and also good selectivity for critical PAH pairs or groups, including the anthracene/phenanthrene pair or benzofluoranthenes. Conduct proper inlet and column maintenance to ensure adequate operation of the GC instrument. Perform system checks.

(**b**) *MS conditions.*—Any GC/MS instrument (single quadrupole, triple quadrupole, TOF, or ion trap) with EI may be used as long as it provides results meeting the laboratory qualification requirements. The 10 study participants used the following instruments: single quadrupole (Agilent 5973–Laboratory 4; Agilent 5975B XL Inert–Laboratories 3 and 8–10; Agilent 5975C–Laboratories 6 and 7), triple quadrupole (Agilent 7000B–Laboratory 5; Thermo TSQ–Laboratory 1), and time-of-flight (Leco Pegasus 4D–Laboratory 2). Pay special attention to the optimization of the MS transfer line and MS source temperature. Higher MS source temperatures

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Table 2014.08G. GC conditions used by collaborative study participants

Laboratory No.	1	2	3	4	5
GC Instrument	Thermo Trace GC Ultra	Agilent 6890N	Agilent 6890N	Agilent 6890	Agilent 7890A
Inlet type	PTV (Thermo 816, RP - 2004)	PTV (Gerstel CIS 4)	S/SL (Agilent)	PTV (Gerstel CIS 4)	Multi-mode (Agilent)
Column type	Thermo TR-50MS	Restek Rxi-17Sil MS	Agilent J&W DB-17MS	Restek Rxi-17Sil MS	Restek Rxi-17Sil MS
Column dimension	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm
Retention gap dimension	NA	NA	NA	2 m × 0.25 mm id, 0.25 µm	NA
Run time, min	40	49.97	47.17	46	39.33
Oven temperature program	60°C (1 min), 12°C/min to 210°C, 8°C/min to 340°C (10 min)	50°C (4.3 min), 30°C/min to 220°C, 2°C/min to 240°C, 5°C/min to 360°C (6 min)	80°C (1 min), 30°C/min to 220°C, 2°C/min to 270°C (1.5 min), 5°C/min to 320°C	65°C (4 min), 30°C/min to 220°C (0.5 min), 3°C/min to 320°C (5 min)	65°C (0.5 min), 15°C/min to 220°C, 4°C/min to 330°C (1 min)
He flow/pressure program	1.2 mL/min (constant flow)	1.3 mL/min (constant flow)	1.3 mL/min (constant flow)	1.3 mL/min (15 min), 50 mL/min^2 to 2 mL/min	2 mL/min (constant flow)
Injection mode	PTV solvent split	PTV solvent vent	Pulsed splitless	PTV solvent vent (stop flow)	PTV solvent vent
Injection volume, µL	8	8	1	8	5
Inlet temperature (program)	50°C (2.3 min), 6.7°C/s to 300°C (1 min), 10°C/s to 350°C (10 min)	50°C (2.3 min), 12°C/s to 340°C	320°C	15°C (0.5 min), 12°C/min to 275°C	70°C (0.15 min), 600°C/min to 325°C (5 min)
Liner	Thermo, LVI Silcosteel, 2 mm id	Gerstel, CIS4 deactivated baffled liner, 2 mm id	Restek, single-gooseneck splitless, 4 mm id	Gerstel, CIS4 deactivated baffled liner, 2 mm id	Agilent, dimpled deactivated, 2 mm id
PTV solvent vent time	0.1 min	138 s	NA	0.5 min	0.1 min
PTV solvent vent flow, mL/min	50	50	NA	100	50
PTV solvent vent pressure, psi	NA	50	NA	1.80	5
Pressure pulse and duration	NA	NA	50 psi for 0.2 min	NA	NA
Split vent purge flow and start	50 mL/min, 1 min	20 mL/min	50 mL/min, 1 min	50 mL/min, 2.5 min	60 mL/min, 2.6 min
Gas saver flow and start time	15 mL/min, 3 min	NA	15 mL/min, 6 min	30 mL/min, 3 min	NA
MS transfer line temperature, °C	250	280	280	280	300
	6	7	8	9	10
GC instrument	Agilent 7890	Agilent 7890	Agilent 6890N	Agilent 6890N	Agilent 6890N
Inlet type	S/SL (Agilent)	S/SL (Agilent)	S/SL (Agilent)	PTV (Apex, ProSep 800 Plus)	PTV (Gerstel CIS 4)
Column type	Restek Rxi-17Sil MS	Restek Rxi-17Sil MS	Agilent J&W DB-EUPAH	Phenomenex ZB-50	Agilent J&W DB-EUPAH
Column dimension	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	20 m × 0.18 mm id, 0.14 µm	30 m × 0.25 mm id, 0.25 µm	20 m × 0.18 mm id, 0.14 µm
Retention gap dimension	1 m × 0.25 mm	NA	NA	NA	NA
GC run time, min	46.33	41.63	31.83	48.27	36.17
Oven temperature program	65°C (0.5 min), 15°C/min to 220°C, 4°C/min to 330°C (8 min)	80°C (4.3 min), 30°C/min to 220°C, 2°C/min to 240°C, 5°C/min to 300°C (1 min), 30°C/min to 350°C (8 min)	80°C (1 min), 30°C/min to 130°C, 12°C/min to 240°C, 3.5°C/min to 310°C	80°C (4.6 min), 30°C/min to 130°C (5 min), 10°C/min to 260°C (15 min), 10°C/min to 320°C (3 min); Post run: 80°C (1 min)	55°C (3 min), 30°C/min to 220°C, 2°C/min to 240°C, 3°C/min to 285°C; 15°C/min to 310°C (1 min); Post run: 330°C (2 min)

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Table 2014.08G. (*continued*)

Laboratory No.	6	7	8	9	10
He flow/pressure program	1.5 mL/min (13 min), 10 mL/min² to 2 mL/min	1.5 mL/min (constant flow)	1 mL/min (constant flow)	1.3 mL/min (19.5 min), 50 mL/min² to 2 mL/min	2 mL/min (constant flow)
Injection mode	Hot splitless	Hot splitless	Pulsed splitless	Splitless	PTV solvent vent
Injection volume, µL	1	1	1	10	5
Inlet temperature (program)	300°C	280°C	320°C	60°C (2.25 min), 300°C/min to 335°C (45 min)	50°C (0.5 min), 600°C/min 400°C (15 min), 30°C/min 70°C
Liner	SGE, Focus liner	Agilent, single taper, glass wool-packed, 4 mm id	Agilent, double taper, 4 mm id	Apex, ProSep taper liner, 2 mm id	Agilent, PTV multi-baffle, 2 mm id
PTV solvent vent time	NA	NA	NA	NA	0.5 min
PTV solvent vent flow	NA	NA	NA	NA	100 mL/min
PTV solvent vent pressure	NA	NA	NA	NA	50 kPa
Pressure pulse and duration	NA	NA	50 psi for 0.2 min	NA	NA
Split vent purge flow and start	50 mL/min, 1 min	50 mL/min, 1 min	50 mL/min, 1 min	50.0 mL/min, 2.30 min	100 mL/min, 3 min
Gas saver flow and start time	20 mL/min, 3 min	20 mL/min, 2 min	15 mL/min, 6 min	NA	15 mL/min, 6 min
MS transfer line temperature, °C	300	280	320	300	325

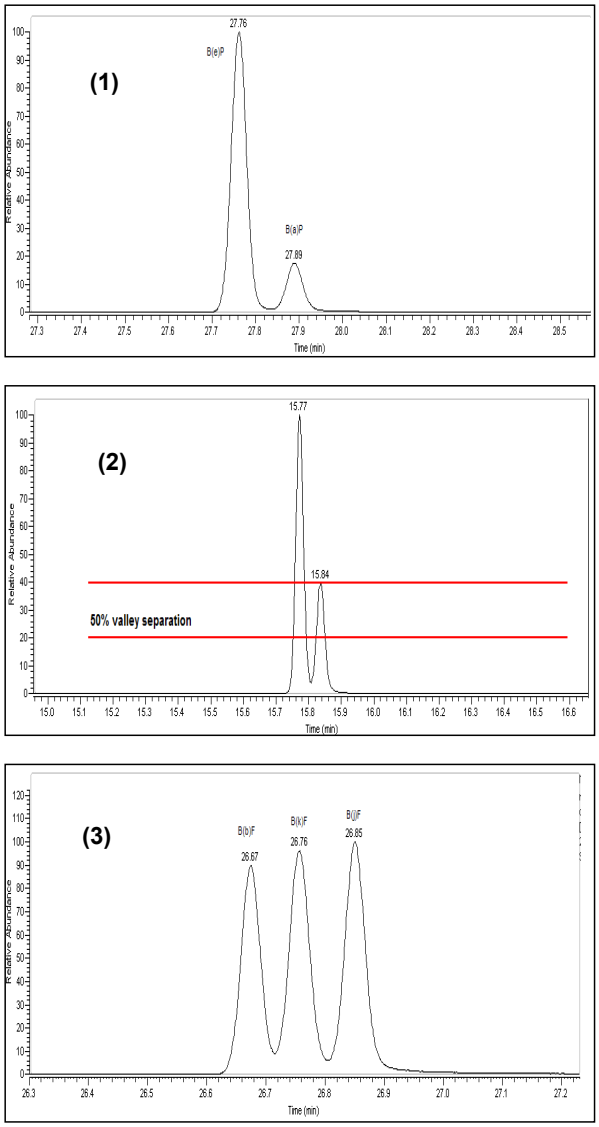

Figure 2014.08C. GC separation criteria: (1) A baseline separation of benzo[*a*]pyrene and benzo[*e*]pyrene (concentration ratio of 1:5), (2) at least 50% valley separation of phenanthrene and anthracene (concentration ratio 2.5:1; evaluated for the anthracene peak, which is the second peak in the figure), and (3) at least 50% valley separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*] fluoranthene (concentration ratio of 1:1:1).

are recommended (e.g., ≥280°C for Agilent sources) to provide optimum analysis (quantitative transfers, minimum peak tailing) for less volatile PAHs.

Table **2014.08H** provides MS ions (*m/z*) and MS/MS transitions used by the study participants for quantification and identification of target PAHs and ^{13}C-PAHs using single-stage MS (single quadrupole and TOF) and tandem MS/MS (triple quadrupole) instruments, respectively.

Use adequate data acquisition rate (dwell times in scanning instruments) and solvent delay time. Perform air/water checks and autotune to verify and obtain adequate operation of the instrument. Verify identification of the analyte peaks by comparing the ion

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Table 2014.08H. MS ions (*m/z*) and MS/MS precursor to product ion transitions used by study participants for quantification (quant) and identification (qual) of target PAHs and ^{13}C-PAHs using single-stage MS (single quadrupole and TOF) and tandem MS/MS (triple quadrupole) instruments, respectively

	Single quad/TOF MS		Triple quad MS/MS	
Compound	Quant	Qual	Quant	Qual
1,7-DMP	206	191	206>190	206>205, 206>165
1-MN	142	115	142>115	142>141, 142>116
1-MP	192	189	192>191	192>165
2,6-DMN	156	141, 144	156>115	156>141
3-MC	242	241	242>239	242>226
Ant	178	177	178>176	178>177, 178>151
BaA	228	226	228>226	228>224, 228>202
BaP	252	253	252>250	250>248, 252>224
BbF	252	253	252>250	250>248, 252>224
BghiP	276	277	276>274	274>272, 276>275
BkF	252	253	252>250	250>248, 252>224
Chr	228	226	228>226	228>224, 228>202
DBahA	278	276	278>276	276>274, 278>274
Fln	166	165	166>165	166>164, 166>163
Flt	202	200	202>200	202>201
IcdP	276	277	276>274	274>272, 276>248
Naph	128	127	128>102	128>127
Phe	178	177	178>176	178>177, 178>151
Pyr	202	200	202>200	202>201
13C-Ant	184	183	184>183	184>182, 184>156
13C-BaA	234	232	234>232	234>206
13C-BaP	256	257	256>254	256>228
13C-BbF	258	259	258>256	258>255
13C-BghiP	288	289	288>286	288>287
13C-BkF	258	259	258>256	258>255
13C-Chr	234	232	234>232	234>206
13C-DBahA	284	282	284>282	284>280
13C-Fln	172	171	172>171	172>170
13C-Flt	208	205	208>206	208>207
13C-IcdP	282	283	282>280	282>281
13C-Naph	134	133	134>133	134>105
13C-Phe	184	183	184>183	184>156
13C-Pyr	205	203, 206, 208	205>203	205>204

ratios of contemporaneously analyzed calibration standards, which have been analyzed under the same conditions.

(**c**) *Injection sequence.*—Bracket the seven test samples with two sets of calibration standards. Inject solvent blanks after the calibration level 8 (highest) standard and after the samples. In addition, analyze a reagent blank with each set of samples. Inject only once from each vial, thus preventing potential losses of volatile PAHs and/or contamination.

H. Calculations

Quantification is based on linear least-squares calibration of analyte signals (S_{PAH}) divided by signals ($S_{13C\text{-}PAH}$) of corresponding ^{13}C-labeled internal standards (*see* Table **2014.08I**) plotted versus analyte concentrations. Peak areas are generally preferred as signals used for the quantification, but peak heights should be used for peaks that are not well resolved, such as in the case of anthracene and phenanthrene. The analyte concentrations in the final extract (c_{PAH}, µg/L) are determined from the equation:

$$c_{PAH} = [(S_{PAH}/S_{13C\text{-}PAH}) - b]/a$$

where a is the slope of the calibration curve and b is the y-intercept.

The concentration of PAHs in the sample (C, µg/kg) is then calculated:

$$C = (c_{PAH}/c_{13C\text{-}PAH}) \times (X_{13C\text{-}PAH}/m)$$

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Table 2014.08I. PAH analytes and corresponding ^{13}C-PAHs used for PAH signal normalization

Analyte	^{13}C-PAH used for signal normalization
Anthracene	Anthracene ($^{13}C_6$)
Benz[*a*]anthracene	Benz[*a*]anthracene ($^{13}C_6$)
Benzo[*a*]pyrene	Benzo[*a*]pyrene ($^{13}C_4$)
Benzo[*b*]fluoranthene	Benzo[*b*]fluoranthene ($^{13}C_6$)
Benzo[*g,h,i*]perylene	Benzo[*g,h,i*]perylene ($^{13}C_{12}$)
Benzo[*k*]fluoranthene	Benzo[*k*]fluoranthene ($^{13}C_6$)
Chrysene	Chrysene ($^{13}C_6$)
Dibenz[*a,h*]anthracene	Dibenz[*a,h*]anthracene ($^{13}C_6$)
Fluoranthene	Fluoranthene ($^{13}C_6$)
Fluorene	Fluorene ($^{13}C_6$)
Indeno[1,2,3-*cd*]pyrene	Indeno[1,2,3-*cd*]pyrene ($^{13}C_6$)
Naphthalene	Naphthalene ($^{13}C_6$)
Phenanthrene	Phenanthrene ($^{13}C_6$)
Pyrene	Pyrene ($^{13}C_6$)
1-Methylnaphthalene	Naphthalene ($^{13}C_6$)
2,6-Dimethylnaphthalene	Phenanthrene ($^{13}C_6$)
1-Methylphenanthrene	Phenanthrene ($^{13}C_6$)
1,7-Dimethylphenanthrene	Phenanthrene ($^{13}C_6$)
3-Methylchrysene	Chrysene ($^{13}C_6$)

where $c_{13C\text{-}PAH}$ is the concentration of the corresponding ^{13}C-PAH in the calibration standard solutions (in µg/L); $X_{13C\text{-}PAH}$ is the amount of the corresponding ^{13}C-PAH added to the sample (in ng); and m is the sample weight (in g). Based on the method procedure and preparation of the calibration standard solutions, $c_{13C\text{-}PAH}$ is 50 µg/L, $X_{13C\text{-}PAH}$ is 50 ng, and m for the test samples is 10 g.

In the collaborative study, eight concentration levels were used for the calibration, corresponding to 5, 10, 20, 50, 100, 200, 500, and 1000 µg/L for benzo[*a*]pyrene and other lower-level PAHs, to 12.5, 25, 50, 125, 250, 500, 1250, and 2500 µg/L for higher-level PAHs, except for naphthalene that was present at 25, 50, 100, 250, 500, 1000, 2500, and 5000 µg/L. Coefficients of determination (r^2) should be 0.990 or greater and back-calculated concentrations of the calibration standards should not exceed ±20% of theoretical. For lower concentration levels, a limited calibration curve (without the higher-end concentration points) may be used for better accuracy. If a well-characterized quadratic relationship occurs, then a best-fitted quadratic curve may be used for calibration. Otherwise, if the back-calculated concentrations exceed ±20% of theoretical, normalized signals of the nearest two calibration standards that enclose the analyte signal in the sample can be used to interpolate the analyte concentration in the final extract.

References: (1) Kalachova, K., Pulkrabova, J., Drabova, L., Cajka, T., Kocourek, V., & Hajslova, J. (2011) *Anal. Chim. Acta* **707**, 84–91. http://dx.doi.org/10.1016/j.aca.2011.09.016

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Candidates for 2016 Method of the Year

RESIDUES AND TRACE ELEMENTS

Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood Using Gas Chromatography-Mass Spectrometry: Collaborative Study

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A collaborative study was conducted to determine selected polycyclic aromatic hydrocarbons (PAHs) and their relevant alkyl homologs in seafood matrixes using a fast sample preparation method followed by analysis with GC/MS. The sample preparation method involves addition of ^{13}C-PAH surrogate mixture to homogenized samples and extraction by shaking with a water–ethyl acetate mixture. After phase separation induced by addition of anhydrous magnesium sulfate–sodium chloride (2 + 1, w/w) and centrifugation, an aliquot of the ethyl acetate layer is evaporated, reconstituted in hexane, and cleaned up using silica gel SPE. The analytes are eluted with hexane–dichloromethane (3 + 1, v/v), the clean extract is carefully evaporated, reconstituted in isooctane, and analyzed by GC/MS. To allow for the use of various GC/MS instruments, GC columns, silica SPE cartridges, and evaporation techniques and equipment, performance-based criteria were developed and implemented in the qualification phase of the collaborative study. These criteria helped laboratories optimize their GC/MS, SPE cleanup, and evaporation conditions; check and eliminate potential PAH contamination in their reagent blanks; and become familiar with the method procedure. Ten laboratories from five countries qualified and completed the collaborative study, which was conducted on three seafood matrixes (mussel, oyster, and shrimp) fortified with 19 selected PAH analytes at five different levels of benzo[*a*]pyrene (BaP) ranging from 2 to 50 µg/kg. Each matrix had a varying mixture of three different BaP levels. The other studied PAHs were at varying levels from 2 to 250 µg/kg to mimic typical PAH patterns. The fortified analytes in three matrixes were analyzed as blind duplicates at each level of BaP and corresponding other PAH levels. In addition, a blank with no added PAHs for each matrix was analyzed singly. Eight to 10 valid results were obtained for the majority of determinations. Mean recoveries of all tested analytes at the five different concentration levels were all in the range of 70–120%: 83.8–115% in shrimp, 77.3–107% in mussel, and 71.6–94.6% in oyster, except for a slightly lower mean recovery of 68.6% for benzo[*a*]anthracene fortified at 25 µg/kg in oyster (RSD_r: 5.84%, RSD_R: 21.1%) and lower mean recoveries for anthracene (Ant) and BaP in oyster at all three fortification levels (50.3–56.5% and 48.2–49.7%, respectively). The lower mean recoveries of Ant and BaP were linked to degradation of these analytes in oyster samples stored at –20°C, which also resulted in lower reproducibility (RSD_R values in the range of 44.5–64.7% for Ant and 40.6–43.5% for BaP). However, the repeatability was good (RSD_r of 8.78–9.96% for Ant and 6.43–11.9% for BaP), and the HorRat values were acceptable (1.56–1.94 for Ant and 1.10–1.45 for BaP). In all other cases, repeatability, reproducibility, and HorRat values were as follows: shrimp: RSD_r 1.40–26.9%, RSD_R 5.41–29.4%, HorRat: 0.22–1.34; mussel: RSD_r 2.52–17.1%, RSD_R 4.19–32.5%, HorRat: 0.17–1.13; and oyster: RSD_r 3.12–22.7%, RSD_R 8.41–31.8%, HorRat: 0.34–1.39. The results demonstrate that the method is fit-for-purpose to determine PAHs and their alkyl homologs in seafood samples. The method was approved by the Expert Review Panel on PAHs as the AOAC Official First Action Method 2014.08.

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The method was approved by the Expert Review Panel for Polycyclic Aromatic Hydrocarbons (PAHs) as First Action.
The Expert Review Panel for Polycyclic Aromatic Hydrocarbons (PAHs) 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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As a response to the 2010 oil spill in the Gulf of Mexico, AOAC INTERNATIONAL formed the Stakeholder Panel on Seafood Contaminants (SPSC) and later issued a call for methods for determination of polycyclic

Table 1. PAHs included in the collaborative study

Name	Abbreviation
1,7-Dimethylphenanthrene	1,7-DMP
1-Methylnaphthalene	1-MN
1-Methylphenanthrene	1-MP
2,6-Dimethylnaphthalene	2,6-DMN
3-Methylchrysene	3-MC
Anthracene	Ant
Benz[*a*]anthracene	BaA
Benzo[*a*] pyrene	BaP
Benzo[*b*]fluoranthene	BbF
Benzo[*g,h,i*]perylene	BghiP
Benzo[*k*]fluoranthene	BkF
Chrysene	Chr
Dibenz[*a,h*]anthracene	DBahA
Fluoranthene	Flt
Fluorene	Fln
Indeno[1,2,3-*cd*]pyrene	IcdP
Naphthalene	Naph
Phenanthrene	Phe
Pyrene	Pyr

aromatic hydrocarbons (PAHs) in seafood. The primary goal was to significantly reduce the time-to-signal (including sample preparation and extraction) in comparison with currently accepted analytical methods requiring 96–120 hours to complete. In addition, acceptable methods had to demonstrate an LOQ of 1 µg/kg for benzo[*a*]pyrene (BaP) in seafood. The SPSC PAH Working Group on Quantitative Methods evaluated about 30 methods that were submitted as a response to the call or found in the literature. The evaluation criteria included: fitness-for-purpose requirements (LOQ, speed, and scope), identification and quantification (compatibility with MS), quality of data to meet AOAC INTERNATIONAL single-laboratory validation requirements (e.g., accuracy, precision, and analysis of reference materials), and practical considerations, such as availability of equipment.

The Working Group selected a method developed for the determination of PAHs, polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) in fish and seafood by Jana Hajslova's group at the Institute of Chemical Technology (ICT) in Prague, Czech Republic (1) within a European integrated project CONffIDENCE (Contaminants in Food and Feed: Inexpensive Detection for Control of Exposure; 2). This method was studied within the presented collaborative study, for which the analytes were narrowed down to include only PAHs and some of the relevant PAH alkyl homologs (*see* Table 1 for the list of 19 studied analytes and their abbreviations).

Table 2. PAH fortification levels (in µg/kg) in the shrimp, mussel, and oyster test samples

	Shrimp			Mussel			Oyster		
	Low	Mid	High	Low	Mid	High	Low	Mid	High
PAH	Level 1	Level 2	Level 4	Level 1	Level 3	Level 4	Level 2	Level 3	Level 5
1,7-DMP [a]	20	20	20	40	40	40	80	80	80
1-MN	20	75	200	20	100	200	75	100	250
1-MP	10	25	125	10	50	125	25	50	200
2,6-DMN	15	40	175	15	75	175	40	75	225
3-MC	10	30	145	10	90	145	30	90	225
Ant	5	10	40	5	15	40	10	15	60
BaA	5	15	60	5	25	60	15	25	100
BaP	2	5	25	2	10	25	5	10	50
BbF	5	10	75	5	30	75	10	30	100
BghiP	2	5	20	2	10	20	5	10	25
BkF	2	8	40	2	20	40	8	20	75
Chr	15	50	175	15	100	175	50	100	250
DBahA	2	5	15	2	10	15	5	10	20
Fln	5	15	50	5	25	50	15	25	75
Flt	10	25	100	10	50	100	25	50	150
IcdP	2	5	20	2	10	20	5	10	25
Naph	25	80	160	25	125	160	80	125	225
Phe	15	50	175	15	100	175	50	100	250
Pyr	15	40	125	15	75	125	40	75	200

[a] 1,7-DMP served as a homogenization check, which was added to the blank mussel and oyster matrix during the homogenization step (prior to fortification).

Collaborative Study

Purpose

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 an Official Method for the determination of PAHs in seafood.

Study Design

This study evaluated the method performance for determination of 19 selected PAHs, including alkyl homologs relevant to an oil spill contamination (*see* Table 1), in three seafood matrixes: shrimp, oysters, and mussels, with five different levels of BaP ranging from 2 to 50 µg/kg. Each matrix had a varying mixture of three different BaP levels ("low," "mid," and "high"). The other studied PAHs were added at varying levels from 2 to 250 µg/kg to mimic typical PAH patterns (Table 2). The fortified analytes in the three matrixes were analyzed as blind duplicates at each level of BaP and corresponding other PAH levels. In addition, a blank with no added PAHs for each matrix was analyzed singly. The AOAC official method guidelines for collaborative study procedures (3) were followed for the preparation of the study and data analysis.

Test Sample Preparation

Blank mussel and oyster samples were homogenized with liquid nitrogen and tested in duplicate by an independent laboratory for potential contamination with the target PAHs. During homogenization, portions of the blank matrixes were spiked with 1,7-dimethylphenanthrene (1,7-DMP) at 40 and 80 µg/kg in the case of mussel and oyster, respectively. These were utilized as a homogenization check throughout the course of the study. The collaborators determined 1,7-DMP along with the other 18 analytes, which were spiked into 10 g sample portions placed in polypropylene centrifuge tubes by the study direction team. Five different spiking levels were made at varying PAH concentrations (Table 2), resulting in three different duplicate spiked samples/matrix in addition to a blank. Participants were supplied with the test samples ready for analysis labeled with unique identification numbers. All test samples were shipped frozen on dry ice with a material receipt document to be returned to the Study Directors. The test samples had to be stored in a freezer set to maintain at least –20 ± 10°C. Test samples were to be analyzed after completion of laboratory qualification and practice sample analysis.

Blank shrimp matrix (peeled, without head and tail, and uncooked) was homogenized without the use of liquid nitrogen using a blender. After testing for potential contamination with the target PAHs, 10 g blank sample portions were placed in polypropylene centrifuge tubes, which were sent to study participants together with spiking solutions labeled with unique identification numbers. Using instructions provided by the Study Directors, participants fortified the blank shrimp samples themselves on the day of the analysis.

Three different spiking levels were used at varying PAH concentrations (Table 2), resulting in three different duplicate spiked samples in addition to a blank (seven samples altogether). The blank shrimp samples were shipped frozen on dry ice with a material receipt document to be returned to the Study Directors. The test samples had to be stored in a freezer set to maintain at least –20 ± 10°C. The spiking solutions were to be stored in a refrigerator set to maintain 5 ± 3°C. (*Note*: This modification of the shrimp test sample preparation protocol (as compared to mussel and oyster) was made (after consultations with the SPSC PAH Working Group and the AOAC Methods Committee on PAHs) due to potential stability issues discovered during the practice sample analysis and follow-up experiments with fortified shrimp samples stored at different conditions. 3-Methylchrysene (3-MC) had to be replaced by 6-methylchrysene (6-MC) in the spiking and calibration solutions for shrimp samples due to the unavailability of a 3-MC reference standard at the time of preparation and shipment of the new set of shrimp samples to the study participants.)

Laboratory Qualification

During the laboratory qualification phase, the collaborators conducted the following seven steps. These steps were necessary because the Study Directors allowed the use of various GC/MS instruments, GC columns, silica SPE cartridges, and evaporation techniques and equipment. Therefore, performance-based criteria were developed to help laboratories optimize their GC/MS, SPE cleanup, and solvent evaporation conditions; check and eliminate potential PAH contamination in their reagent blanks; and become familiar with the method. Laboratory qualification and practice sample results had to be approved by the Study Directors before proceeding with the test sample analysis. Sixteen laboratories entered the qualification phase, but only 10 of them (listed in the *Acknowledgments* section) completed the qualification successfully and/or continued in the study.

(*1*) The first step was a GC separation test where participants analyzed a composite PAH solution by GC/MS/MS to obtain a baseline separation of BaP and benzo[*e*]pyrene (concentration ratio of 1:5); at least 50% valley separation of anthracene and phenanthrene (concentration ratio 1:2.5, evaluated for the anthracene peak); and at least 50% valley separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*]fluoranthene (concentration ratio of 1:1:1).

(*2*) The second step was a calibration range test where participants prepared calibration standards and obtained normalized calibration curves for the studied PAHs versus respective labeled internal standards (^{13}C-PAHs). Collaborators had to determine the linear range, test for carryover by injecting a solvent blank after the highest standard, and adjust injection conditions (such as injection volume, number of washes, syringe size, etc.) to achieve low detection limits, acceptable linearity for the tested concentration range, and minimum carryover. Coefficient of determination (r^2) values should be 0.990 or greater, and back-calculated concentrations of the calibration standards should not exceed ±20% of theoretical. For lower concentration levels, a limited calibration curve (without the higher-end concentration points) may be used for better accuracy. If a well characterized quadratic relationship occurs, then a best-fitted quadratic curve may be used for calibration. Otherwise, if the back-calculated concentrations exceed ±20% of theoretical, normalized signals of the nearest two calibration

standards that enclose the analyte signal in the sample could be used to interpolate the analyte concentration.

(*3*) The third step was a test of the solvent evaporation where participants determined absolute recoveries of both PAHs and ^{13}C-PAHs in two evaporation experiments (with three replicates each): (**a**) gentle evaporation of 5 mL of a PAH/^{13}C-PAH solution in ethyl acetate and reconstitution in isooctane and (**b**) gentle evaporation of 10 mL of a PAH/^{13}C-PAH solution in hexane–dichloromethane (3 + 1, v/v) and reconstitution in isooctane. The absolute recoveries of all analytes, including naphthalene, and ^{13}C-naphthalene had to be above 70%.

(*4*) The fourth step was the determination of the elution profiles of PAHs and fat on silica gel SPE columns chosen for the PAH analysis by the laboratory. The silica gel columns could be prepared in-house using the procedure described in the method or could be obtained commercially from different vendors. The elution volume of 10 mL hexane–dichloromethane (3 + 1, v/v) specified in the ICT method (1) was optimized for the analysis of PAHs, PCBs, and PBDEs using the in-house prepared silica gel minicolumns for which the silica gel deactivation (5% water added) and storage are controlled by the laboratory. For commercially available silica gel SPE cartridges, however, the deactivation and storage can vary, potentially resulting in different amounts of water in the silica thus potentially different retention characteristics. Therefore, it is important to test the elution profiles of PAHs and fat and determine the optimum volume of the elution solvent to ensure adequate analyte recoveries and fat cleanup. The PAH elution profile was determined by applying 1 mL of a PAH in hexane solution to the silica cartridge, collecting fractions of hexane–dichloromethane (3 + 1, v/v) eluting from the cartridge, exchanging the fractions to 0.5 mL isooctane, and analyzing them by GC/MS. The fat elution profile was checked gravimetrically by applying 1 mL of hexane containing 100 mg of fat (pure fish oil) onto the silica cartridge, collecting the optimum elution fraction determined for PAHs and three consecutive 1 mL fractions, and evaporating them to dryness.

(*5*) The fifth step was a reagent (procedure) blank test where participants determined concentrations of the target PAHs in three replicates of reagent (procedure) blank that was prepared the same way as the samples, except that 10 mL of water was used instead of the sample. The concentrations of all analytes in the reagent blanks had to be below the concentrations in the lowest calibration level standard. For naphthalene, levels below the second lowest calibration standard (equivalent to 5 ng/g of naphthalene in the sample) were still acceptable if the source of contamination could not be eliminated, such as by selection of a silica gel SPE column from a different vendor (or preparation of silica gel columns in-house), heating of glassware, addition of a hydrocarbon trap to the nitrogen lines used for solvent evaporation, etc.

(*6*) The sixth step was a low-level spike test where collaborators prepared and analyzed seven spiked samples using blank shrimp matrix and a mixed PAH spiking solution that were both supplied to them. The samples were spiked at PAH concentrations equivalent to the second lowest calibration level (1 µg/kg for BaP, which is a fitness-for-purpose LOQ requirement established for the study) to test instrument sensitivity and method precision. The shrimp matrix had to be stored in a freezer set to maintain at least –20 ± 10°C. The mixed PAH spiking solution was to be stored in a refrigerator set to maintain 5 ± 3°C.

(*7*) The seventh step was the analysis of practice samples. Three practice samples were supplied to the participants. Two of the three samples were shrimp blank matrix already spiked with two different mixed PAH solutions (BaP levels of 2–50 µg/kg, other PAHs at 2–250 µg/kg). The third sample was the National Institute of Standards and Technology Standard Reference Material 1974b, which is a mussel matrix with certified concentrations of incurred PAHs and other organic contaminants. All practice samples were shipped frozen on dry ice and had to be stored in a freezer set to maintain at least –20 ± 10°C.

Quality Assurance

The method uses a mixture of isotopically labeled ^{13}C-PAH surrogate standards that were added at 5 µg/kg to the samples prior to the extraction process. Quantification was based on calibration of analyte signals (peak areas or heights) divided by signals of respective ^{13}C-labeled internal standards plotted versus analyte concentrations. Eight concentration levels were used for the calibration, corresponding to 0.5, 1, 2, 5, 10, 20, 50, and 100 µg/kg for BaP and other lower level PAHs, and to 1.25, 2.5, 5, 12.5, 25, 50, 125, and 250 µg/kg for higher level PAHs, except for naphthalene that was present at levels corresponding to 2.5, 5, 10, 25, 50, 100, 250, and 500 µg/kg. Values of r^2 had to be 0.990 or greater, and back-calculated concentrations of the calibration standards should not exceed ±20% of theoretical. For lower concentration levels, a limited calibration curve (without the three higher-end concentration points) was used for better accuracy. In addition to reporting r^2 values, back-calculated calibration standard concentrations, and analyte concentrations, the collaborators were also required to report ion ratios as a means of verifying identification of the analyte peaks.

A solvent (isooctane) blank was injected before and after each calibration set. Reagent (procedural) blanks were analyzed with each set of samples. During homogenization, portions of the blank mussel and oyster matrixes were spiked with 1,7-DMP, which served as a homogenization check of the sample processing step.

Data Reporting

Participants supplied PAH and ^{13}C-PAH signals (peak areas or heights) in test samples, calibration standards, and blanks and other parameters as described above in Quality Assurance in Excel forms created by the Study Directors. They also had to provide details about their GC and MS instruments and method conditions, evaporation equipment and conditions, and silica gel SPE cartridge and optimum elution volume. Participants were asked to record all observations and any potential method deviations, investigate any potential unreasonable results (caused by, e.g., incorrect calculations and arithmetic errors, use of wrong units, transposition errors, incorrect standard preparation or contamination), and have all the results and

calculations reviewed by a peer, laboratory supervisor, or manager.

Data Analysis

The Study Directors reviewed and compiled all the data submitted by the participants. Statistical analysis was conducted using the AOAC spreadsheet for blind duplicates (4) to determine mean analyte concentrations, SD (S_r) and RSD (RSD_r) for repeatability (for blind duplicate data), SD (S_R) and RSD (RSD_R) for reproducibility, number of valid data points, HorRat value (RSD_R/predicted RSD_R), and percentage recovery for all data after removal of outliers (3). The following tests were used in the AOAC spreadsheet (4) to determine outliers: (*a*) the Cochran test for removal of laboratories showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material, and (*b*) the Grubbs' tests for removal of laboratories with extreme averages.

AOAC Official Method 2014.08
Polycyclic Aromatic Hydrocarbons (PAHs) in Seafood
Gas Chromatography-Mass Spectrometry
First Action 2014

[Applicable for the determination of the following PAHs in mussel, oyster, and shrimp: 1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene. These were representative PAH analytes selected for the collaborative study. The method has been single-laboratory validated for 32 PAHs in fish and shrimp (1), and, therefore, is expected to be applicable to other GC-amenable PAHs and seafood matrices. The concentration ranges evaluated within the collaborative study are given in Table **2014.08A**.]

Caution: *See Appendix B: Laboratory Safety*. Use appropriate personal protective equipment such as laboratory coat, safety glasses or goggles, appropriate chemical-resistant gloves, and a fume hood. Dispose of solvents and solutions according to federal, state, and local regulations. Always handle open containers of solvents inside the fume hood, including the pouring, mixing, evaporating, and preparing standard solution. Keep containers covered or closed when not in use.

Hexane and isooctane.—Highly flammable, liquid irritants. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation.

Ethyl acetate.—Highly flammable, liquid irritants. Harmful if swallowed in quantity. Vapors may cause drowsiness.

Toluene.—Highly flammable, liquid irritant. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation. May cause drowsiness. Possible teratogen.

Dichloromethane.—Noncombustible, liquid irritant. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation. Asphyxiant. Causes central nervous system (CNS) depression. Possible carcinogen and mutagen.

PAHs.—Carcinogens, respiratory sensitizers, teratogens, reproductive hazard, mutagens. Harmful if inhaled, swallowed, or absorbed through the skin. May also cause skin and eye irritation.

See Tables **2014.08B–D** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Homogenized seafood samples (10 g sample with a 5 µg/kg addition of ^{13}C-PAH surrogate mixture) are mixed with 5 mL water (or 10 mL water in the case of shrimp and other more viscous samples) and shaken vigorously by hand with 10 mL ethyl acetate in a 50 mL polypropylene centrifuge tube for 1 min. Subsequently, 4 g anhydrous magnesium sulfate and 2 g sodium chloride are added to the mixture to induce phase separation and force the analytes into the ethyl acetate layer. The tube is again shaken by hand for 1 min and then centrifuged for 10 min at >1500 rcf. A 5 mL aliquot of the ethyl acetate layer is evaporated, reconstituted in 1 mL hexane, and cleaned on an SPE column with 1 g silica gel and approximately 0.2 g anhydrous sodium sulfate on the top. The column is conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v)

Table 2014.08A. PAH and ^{13}C-PAH concentrations in the calibration standard solutions

	Concentration, µg/L				Equivalent concentration, µg/kg			
Calibration level	BaP and others[a]	Chr and others[b]	Naph[c]	^{13}C-PAHs	BaP and others	Chr and others	Naph	^{13}C-PAHs
1	5	12.5	25	50	0.5	1.25	2.5	5
2	10	25	50	50	1	2.5	5	5
3	20	50	100	50	2	5	10	5
4	50	125	250	50	5	12.5	25	5
5	100	250	500	50	10	25	50	5
6	200	500	1000	50	20	50	100	5
7	500	1250	2500	50	50	125	250	5
8	1000	2500	5000	50	100	250	500	5

[a] Analytes at 10 µg/mL in the mixed stock standard solution.

[b] Analytes at 25 µg/mL in the mixed stock standard solution.

[c] Analytes at 50 µg/mL in the mixed stock standard solution.

Table 2014.08B. Statistical results for the studied PAHs at three different concentration levels in shrimp after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	9	18	21.7	108.6	2.6	4.1	11.8	18.9	0.66
	9	18	22.7	113.7	1.8	4.2	8.0	18.7	0.66
	9	18	21.7	108.3	1.9	4.4	8.8	20.4	0.72
1-MN	9	18	23.1	115.4	6.2	6.8	26.9	29.4	1.04
	9	18	81.5	108.6	6.7	15.6	8.3	19.1	0.82
	9	18	203.2	101.6	17.8	55.4	8.8	27.3	1.34
1-MP	9	18	10.0	99.9	1.0	1.4	9.8	14.0	0.44
	9	18	25.0	99.9	1.9	3.7	7.6	14.9	0.53
	9	18	119.4	95.5	6.7	15.7	5.6	13.1	0.60
2,6-DMN	8	16	15.6	103.9	1.1	2.6	6.9	16.4	0.55
	8	16	37.8	94.6	4.8	7.4	12.6	19.5	0.74
	7	14	146.7	83.8	16.6	20.3	11.3	13.9	0.65
6-MC	9	18	11.1	110.5	0.6	1.5	5.8	13.1	0.42
	9	18	32.2	107.2	1.6	3.6	5.1	11.1	0.42
	9	18	145.2	100.1	8.7	13.7	6.0	9.4	0.44
Ant	9	18	4.9	98.5	0.3	0.5	6.7	10.3	0.29
	9	18	10.6	105.7	0.8	1.7	7.3	16.2	0.51
	9	18	38.9	97.4	2.4	4.6	6.2	11.7	0.45
BaA	9	18	4.8	95.9	0.3	0.5	7.0	9.7	0.27
	9	18	15.0	99.9	0.6	1.3	4.3	8.4	0.28
	9	18	56.6	94.4	2.5	5.2	4.5	9.2	0.37
BaP	9	18	1.9	96.2	0.1	0.2	6.5	12.1	0.29
	9	18	4.9	98.7	0.4	0.5	7.3	9.6	0.27
	8	16	23.1	92.3	1.1	1.6	4.6	7.0	0.25
BbF	9	18	4.8	96.7	0.3	0.5	6.6	10.1	0.28
	9	18	9.8	98.2	0.3	0.7	2.6	7.0	0.22
	9	18	71.6	95.5	3.9	6.3	5.5	8.8	0.37
BghiP	8	16	1.9	94.7	0.1	0.2	7.0	11.7	0.28
	8	16	4.9	98.5	0.2	0.5	4.3	9.9	0.28
	8	16	18.0	90.1	1.0	1.4	5.7	7.9	0.27
BkF	9	18	2.0	99.5	0.1	0.3	6.2	13.7	0.34
	9	18	8.1	101.7	0.4	0.7	4.9	8.7	0.26
	9	18	38.3	95.8	1.8	2.8	4.7	7.2	0.28
Chr	8	16	15.2	101.5	0.5	1.4	3.1	9.4	0.31
	9	18	50.7	101.4	2.2	4.1	4.4	8.1	0.32
	9	18	167.4	95.6	9.0	14.5	5.3	8.7	0.41
DBahA	9	18	1.9	95.9	0.2	0.3	10.9	13.5	0.33
	9	18	5.0	100.4	0.3	0.6	6.6	11.2	0.32
	9	18	13.8	91.8	0.9	1.2	6.4	8.4	0.28
Fln	8	16	5.2	103.0	0.1	0.5	1.4	10.0	0.28
	9	18	15.4	102.3	0.6	1.1	4.2	7.5	0.25
	9	18	47.3	94.7	2.1	4.1	4.5	8.7	0.34
Flt	9	18	9.7	97.2	0.6	1.0	6.0	10.4	0.32
	9	18	25.1	100.3	1.4	2.4	5.5	9.7	0.35
	9	18	93.9	93.9	4.9	8.7	5.2	9.3	0.41
IcdP	9	18	2.0	98.2	0.1	0.3	5.3	13.3	0.32
	9	18	5.1	102.2	0.5	0.6	9.1	11.0	0.31

Table 2014.08B. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	9	18	18.4	92.1	1.1	1.9	5.7	10.5	0.36
Naph	8	16	27.7	110.7	2.8	2.8	10.3	10.3	0.37
	9	18	84.1	105.1	5.6	8.8	6.7	10.5	0.45
	8	16	158.7	99.2	9.7	34.2	6.1	21.6	1.02
Phe	8	16	15.1	100.5	0.5	1.2	3.3	7.8	0.26
	9	18	49.7	99.4	1.5	3.0	3.1	6.0	0.24
	9	18	168.0	96.0	8.6	16.6	5.1	9.9	0.47
Pyr	9	18	14.8	98.5	0.9	1.3	6.1	8.8	0.29
	9	18	40.3	100.8	1.6	3.3	3.8	8.2	0.32
	8	16	118.7	95.0	2.9	6.4	2.5	5.4	0.25

and 4 mL hexane, followed by application of the 1 mL extract in hexane. The analytes are eluted with hexane–dichloromethane (3 + 1, v/v) using volume determined for the given silica gel SPE cartridges from the elution profiles of target analytes and fat, which are dependent on the silica deactivation. The clean extract is carefully evaporated, reconstituted in 0.5 mL isooctane, and analyzed by GC/MS. *See* Figure **2014.08A** for the method flow chart.

B. Apparatus

(**a**) *Homogenizer.*—WARING blender Model 38BL40 (Conair Corp., Stamford, CT) or equivalent.

(**b**) *Solvent evaporator.*—Any suitable solvent evaporator, such as a rotary vacuum evaporator, Kuderna-Danish evaporator, or a nitrogen blow-down system, may be used as long as it provides results meeting the laboratory qualification/method set-up requirements (absolute analyte recoveries >70% in both evaporation steps).

(**c**) *Centrifuge.*—Capable of centrifugation of 50 mL tubes at >1500 rcf for 10 min.

(**d**) *Furnace/oven.*—Capable of 600°C operation.

(**e**) *Balance(s).*—Analytical, capable of accurately measuring weights from 1 mg to 10 g.

(**f**) *Gas chromatograph-mass spectrometer.*—Any GC/MS instrument [single quadrupole, triple quadrupole, time-of-flight (TOF), or ion trap] with electron ionization (EI) may be used as long as it provides results meeting the laboratory qualification requirements (to provide reliable results for the calibration range specified in Table **2014.08A**).

(**g**) *GC column.*—Capillary column BPX-50 (30 m, 0.25 mm id, 250 µm film thickness; Trajan Scientific, Austin, TX, USA) or equivalent (USP specification G3), such as Rxi-17Sil MS (Restek Corp., Bellefonte, PA, USA); DB-17MS, DB-17, or HP-50 (Agilent Technologies, Santa Clara, CA, USA); or any other column that enables adequate separation of PAHs as specified in the laboratory qualification requirements (*see* **G**).

C. Reagents and Materials

(**a**) *Hexane.*—>98.5%, mixture of isomers.

(**b**) *Isooctane.*—ACS or better grade.

(**c**) *Ethyl acetate.*—>99.5%, for GC residue analysis.

(**d**) *Dichloromethane.*—≥99.9%, for GC residue analysis.

(**e**) *Toluene.*—≥99.9%, for GC residue analysis.

(**f**) *Water.*—Purified, free of interfering compounds.

(**g**) *Anhydrous sodium sulfate (Na_2SO_4).*—≥99.0%, powder, heated at 600°C for 7 h and then stored in a desiccator before use (Na_2SO_4 prepared and stored as indicated can be used for 1 month from preparation).

(**h**) *Silica gel SPE column.*—Containing 1 g silica gel. Any commercially available silica gel SPE column can be used as long as it provides adequate fat cleanup and meets requirements for low background contamination specified in the laboratory qualification requirements: the concentrations of all analytes in the reagent blanks had to be below the concentrations in the lowest calibration level standard; for naphthalene, levels below the second lowest calibration standard (equivalent to 5 ng/g naphthalene in the sample) are still acceptable if the source of contamination could not be eliminated.

Silica gel SPE columns can be prepared in-house using the following procedure: Activate the silica gel by heating at 180°C for 5 h, and then deactivate it by adding 5% deionized water, shaking for 3 h. Store in a desiccator for 16 h before use (silica gel prepared and stored as indicated can be used for 14 days). Place a piece of deactivated glass wool in a Pasteur pipet (5 mL), add 1 g activated silica gel (Silica gel 60, 0.063–0.2 mm, 70–230 mesh or equivalent) and top it with approximately 0.2 g muffled anhydrous Na_2SO_4.

(**i**) *Anhydrous magnesium sulfate ($MgSO_4$).*—≥99.0%, powder, heated (muffled) at 600°C for 7 h, and then store in a desiccator before use ($MgSO_4$ prepared and stored as indicated can be used for 1 month from preparation). *Note*: A preweighed (commercially available) mixture of 2 g sodium chloride and 4 g anhydrous magnesium sulfate (muffled) in pouches or tubes can be used.

(**j**) *Sodium chloride (NaCl).*—≥99.0%.

(**k**) *Helium 5.0 or better, nitrogen 4.0 or better.*

(**l**) *Polypropylene centrifuge tubes.*—50 mL.

(**m**) *Glass Pasteur pipet.*—5 mL (for solvent transfers and/or in-house preparation of silica gel minicolumns).

(**n**) *Syringes/pipets.*—Capable of accurate measurement and transfer of appropriate volumes for standard solution preparation and sample fortification (50–1000 µL).

(**o**) *Volumetric flasks.*—5–100 mL.

(**p**) *Glassware for evaporation steps.*—Depending on the

Table 2014.08C. Statistical results for the studied PAHs at three different concentration levels in mussel after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	10	20	38.1	95.3	5.7	8.8	14.9	23.2	0.89
	9	18	39.4	98.6	4.6	8.3	11.7	21.1	0.81
	10	20	38.9	97.3	4.6	8.9	11.9	22.8	0.87
1-MN	10	20	19.3	96.5	3.1	4.5	16.2	23.2	0.80
	10	20	96.9	96.9	9.0	19.2	9.2	19.8	0.87
	8	16	208.7	104.4	26.6	26.6	12.7	12.7	0.63
1-MP	9	18	9.8	98.1	0.7	1.8	7.2	18.0	0.56
	9	18	45.8	91.6	5.3	9.6	11.6	21.0	0.82
	9	18	117.2	93.7	10.2	24.4	8.7	20.8	0.94
2,6-DMN	10	20	13.8	91.9	1.5	2.9	11.2	20.7	0.68
	8	16	65.4	87.2	3.1	13.6	4.8	20.7	0.86
	10	20	153.8	87.9	10.7	36.8	6.9	23.9	1.13
3-MC	10	20	9.9	98.6	0.7	1.7	7.6	16.7	0.52
	10	20	87.2	96.8	4.1	10.9	4.7	12.5	0.54
	10	20	138.0	95.2	3.8	16.7	2.8	12.1	0.56
Ant	10	20	3.9	77.9	0.3	1.3	6.7	32.5	0.88
	9	18	13.0	86.8	1.7	3.2	12.8	24.8	0.81
	9	18	31.5	78.8	1.4	7.9	4.6	25.0	0.93
BaA	10	20	4.3	85.1	0.3	0.7	6.9	15.9	0.44
	10	20	21.5	85.9	1.1	2.3	5.1	10.9	0.38
	8	16	52.6	87.7	1.3	2.2	2.5	4.2	0.17
BaP	9	18	1.6	79.2	0.1	0.3	5.6	20.5	0.49
	9	18	8.1	80.5	0.6	1.4	6.9	17.7	0.54
	9	18	19.3	77.3	0.6	2.6	3.0	13.4	0.46
BbF	10	20	4.7	94.4	0.3	0.5	7.2	10.6	0.30
	10	20	27.1	90.2	1.9	2.8	6.9	10.2	0.37
	10	20	69.1	92.1	2.1	5.8	3.0	8.4	0.35
BghiP	9	18	2.0	98.1	0.1	0.2	4.9	10.6	0.26
	10	20	9.3	92.7	0.4	1.1	4.4	12.1	0.37
	10	20	17.9	89.6	0.6	1.5	3.2	8.6	0.29
BkF	9	18	1.9	97.4	0.1	0.2	7.2	10.3	0.25
	10	20	18.5	92.7	1.1	2.4	6.2	12.8	0.44
	10	20	36.6	91.5	1.0	3.8	2.7	10.4	0.40
Chr	10	20	14.2	94.4	0.8	1.3	5.7	9.5	0.31
	10	20	91.6	91.6	4.1	8.6	4.5	9.4	0.41
	10	20	159.8	91.3	4.9	11.8	3.1	7.4	0.35
DBahA	10	20	1.9	93.1	0.2	0.2	9.1	11.2	0.27
	10	20	9.1	90.5	0.5	1.2	5.4	13.5	0.41
	10	20	13.6	90.8	0.5	1.2	4.0	8.5	0.28
Fln	10	20	5.4	107.7	0.2	0.6	3.5	10.6	0.30
	10	20	25.5	101.8	1.0	1.9	3.7	7.6	0.27
	9	18	48.1	96.2	1.7	2.4	3.5	4.9	0.20
Flt	9	18	10.2	102.4	1.1	1.4	10.7	13.2	0.41
	10	20	48.9	97.7	2.7	4.3	5.5	8.8	0.35
	9	18	93.3	93.3	4.9	6.6	5.3	7.1	0.31
IcdP	10	20	2.0	97.7	0.1	0.2	7.3	11.3	0.28
	10	20	9.4	93.7	0.5	1.1	5.6	11.9	0.37

Table 2014.08C. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	10	20	17.9	89.3	0.9	1.6	4.9	8.8	0.30
Naph	9	18	23.7	94.6	1.9	4.0	8.1	17.1	0.61
	10	20	105.9	84.7	10.1	26.7	9.5	25.2	1.12
	8	16	146.7	91.7	7.2	19.2	4.9	13.1	0.61
Phe	8	16	14.5	96.8	0.8	0.9	5.3	6.1	0.20
	8	16	93.1	93.1	3.9	8.5	4.1	9.2	0.40
	8	16	160.8	91.9	5.8	13.0	3.6	8.1	0.38
Pyr	10	20	14.2	94.6	0.7	1.3	5.0	9.3	0.31
	10	20	71.5	95.4	2.9	7.0	4.0	9.8	0.41
	10	20	116.5	93.2	4.5	9.6	3.8	8.3	0.37

evaporation technique (e.g., small round-bottom flasks, suitable tubes, or glassware for Kuderna-Danish evaporation). It is recommended to heat the glassware for at least 2 h at 250°C to remove potential contamination.

D. Reference Standards

(**a**) *PAH standards.*—High-purity reference standards of the PAH analytes (1,7-dimethylphenanthrene, 1-methylnaphthalene, 1-methylphenanthrene, 2,6-dimethylnaphthalene, 3-methylchrysene, anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*k*]fluoranthene, chrysene, dibenz[*a,h*]anthracene, fluoranthene, fluorene, indeno[1,2,3-*cd*]pyrene, naphthalene, phenanthrene, and pyrene).

(**b**) *U.S. Environmental Protection Agency (EPA) 16 PAH cocktail.*—(^{13}C, 99%), Product No. ES-4087 (5 µg/mL, 1.2 mL in nonane), Cambridge Isotope Labs (Tewksbury, MA, USA) or equivalent.

Containing: Acenaphthene ($^{13}C_6$, 99%), acenaphthylene ($^{13}C_6$, 99%), anthracene ($^{13}C_6$, 99%), benz[*a*]anthracene ($^{13}C_6$, 99%), benzo[*b*]fluoranthene ($^{13}C_6$, 99%), benzo[*k*]fluoranthene ($^{13}C_6$, 99%), benzo[*g,h,i*]perylene ($^{13}C_{12}$, 99%), benzo[*a*]pyrene ($^{13}C_4$, 99%), chrysene ($^{13}C_6$, 99%), dibenz[*a,h*]anthracene ($^{13}C_6$, 99%), fluoranthene ($^{13}C_6$, 99%), fluorene ($^{13}C_6$, 99%), indeno[1,2,3-*cd*]pyrene ($^{13}C_6$, 99%), naphthalene ($^{13}C_6$, 99%), phenanthrene ($^{13}C_6$, 99%), and pyrene ($^{13}C_6$, 99%).

E. Preparation of Standard Solutions

(**a**) *Individual stock solutions.*—Prepare individual PAH stock solutions at approximately 1000 or 2500 µg/mL in toluene.

(**b**) *Mixed stock standard solution.*—Use analyte individual stock solutions to obtain a mixed solution of each PAH at 10 µg/mL (for benzo[*a*]pyrene) and other low-level PAHs) or 25 µg/mL (for chrysene and other higher-level PAHs) or 50 µg/mL (for naphthalene) in isooctane. *See* Table **2014.08E** for analyte concentrations in the mixed stock standard solution.

(**c**) *Working PAH Solution A.*—Accurately transfer 0.5 mL of the mixed stock standard solution into a 5 mL volumetric flask and dilute to volume with isooctane.

(**d**) *Working PAH Solution B.*—Accurately transfer 0.5 mL of the Working PAH Solution A into a 5 mL volumetric flask and dilute to volume with isooctane.

(**e**) *Internal standard solution.*—Prepare 1 µg/mL solution of ^{13}C-PAHs in isooctane by 5-fold dilution of the 5 µg/mL EPA 16 ^{13}C-PAHs cocktail with isooctane.

(**f**) *Calibration standard solutions*—Prepare eight levels of calibration standard solutions (1 mL each) in 2 mL amber screw-cap vials. It is recommended to distribute small portions (enough for a single injection) of the calibration standard solutions into multiple crimp-top vials with 100 µL deactivated glass inserts. *See* Table **2014.08A** for analyte concentrations in the calibration standards and Table **2014.08F** for the dilution scheme.

(*1*) *For level 1 calibration standard.*–Accurately transfer 50 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 900 µL isooctane. Cap the vial and vortex mix briefly.

(*2*) *For level 2 calibration standard.*–Accurately transfer 100 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

(*3*) *For level 3 calibration standard.*–Accurately transfer 200 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 750 µL isooctane. Cap the vial and vortex mix briefly.

(*4*) *For level 4 calibration standard.*–Accurately transfer 500 µL of the Working PAH Solution B into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 450 µL isooctane. Cap the vial and vortex mix briefly.

(*5*) *For level 5 calibration standard.*–Accurately transfer 100 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

(*6*) *For level 6 calibration standard.*–Accurately transfer 200 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 750 µL isooctane. Cap the vial and vortex mix briefly.

(*7*) *For level 7 calibration standard.*–Accurately transfer 500 µL of the Working PAH Solution A into the vial and add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 450 µL isooctane. Cap the vial and vortex mix briefly.

(*8*) *For level 8 calibration standard.*–Accurately transfer 100 µL of the mixed stock standard solution into the vial and

Table 2014.08D. Statistical results for the studied PAHs at three different concentration levels in oyster after elimination of statistical outliers

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
1,7-DMP	8	16	72.3	90.4	4.1	11.7	5.7	16.2	0.68
	8	16	69.0	86.2	4.9	12.6	7.1	18.3	0.76
	8	16	65.6	82.0	5.5	13.2	8.4	20.1	0.83
1-MN	8	16	67.9	90.5	4.3	14.8	6.4	21.8	0.91
	9	18	90.8	90.8	20.6	28.9	22.7	31.9	1.39
	9	18	236.6	94.6	26.4	55.3	11.2	23.4	1.18
1-MP	7	14	20.2	80.8	1.8	4.8	8.8	23.9	0.83
	8	16	39.9	79.8	2.6	7.7	6.4	19.3	0.74
	8	16	154.7	77.3	16.9	34.7	10.9	22.4	1.06
2,6-DMN	7	14	30.5	76.2	3.0	5.3	9.8	17.5	0.65
	7	14	57.9	77.2	4.9	10.8	8.5	18.6	0.76
	7	14	161.3	71.7	12.7	20.9	7.9	13.0	0.62
3-MC	9	18	27.8	92.5	1.7	3.6	6.0	13.0	0.47
	9	18	79.8	88.6	5.1	10.3	6.4	13.0	0.55
	9	18	196.8	87.5	10.1	23.5	5.1	12.0	0.59
Ant	7	14	5.3	53.2	0.5	2.9	8.8	55.0	1.56
	7	14	7.5	50.3	0.8	4.9	10.0	64.7	1.94
	6	12	34.0	56.6	3.0	15.1	8.8	44.5	1.67
BaA	9	18	10.9	72.6	0.8	2.2	7.7	19.7	0.62
	9	18	17.2	68.6	1.0	3.6	5.8	21.1	0.71
	9	18	71.6	71.6	3.9	12.5	5.4	17.5	0.73
BaP	9	18	2.5	49.7	0.3	1.1	11.9	43.5	1.10
	9	18	4.8	48.2	0.4	2.0	9.0	42.2	1.18
	9	18	24.6	49.3	1.6	10.0	6.4	40.5	1.45
BbF	9	18	8.6	85.9	0.6	1.0	6.6	11.6	0.35
	9	18	24.6	81.8	1.7	2.7	7.1	11.2	0.40
	9	18	82.8	82.8	3.4	9.8	4.1	11.9	0.51
BghiP	9	18	4.1	82.4	0.2	0.5	5.9	12.3	0.34
	9	18	8.2	81.9	0.7	1.1	8.6	13.6	0.41
	9	18	19.6	78.4	1.0	2.3	4.9	11.7	0.41
BkF	9	18	6.9	85.9	0.5	1.1	7.7	16.3	0.48
	9	18	16.9	84.3	1.1	2.6	6.5	15.4	0.52
	9	18	62.9	83.8	3.8	8.2	6.1	13.1	0.54
Chr	9	18	43.0	85.9	2.8	4.3	6.5	9.9	0.39
	9	18	81.6	81.6	5.0	8.6	6.2	10.6	0.45
	9	18	204.1	81.6	8.7	19.0	4.3	9.3	0.46
DBahA	9	18	4.1	82.7	0.4	0.5	9.0	13.0	0.35
	8	16	8.2	82.2	0.6	1.1	7.5	13.4	0.41
	9	18	16.0	80.0	0.7	2.0	4.4	12.7	0.43
Fln	9	18	12.5	83.3	1.0	1.9	8.2	15.4	0.50
	9	18	20.3	81.2	1.4	3.0	6.8	14.6	0.51
	9	18	57.0	76.0	2.3	11.3	4.0	19.9	0.81
Flt	9	18	22.0	88.2	2.1	3.6	9.5	16.2	0.57
	9	18	42.0	83.9	5.1	7.5	12.2	17.9	0.69
	9	18	120.7	80.5	7.0	17.1	5.8	14.2	0.65
IcdP	9	18	4.3	86.8	0.4	0.6	9.6	13.1	0.36
	9	18	8.3	83.3	0.8	1.1	9.0	13.7	0.42

Table 2014.08D. (*continued*)

PAH	No. of laboratories	No. of replicates	Mean concn, µg/kg	Mean recovery, %	s_r, µg/kg	s_R, µg/kg	RSD_r, %	RSD_R, %	HorRat
	9	18	20.0	80.1	1.0	2.2	4.8	10.7	0.37
Naph	9	18	71.0	88.7	5.3	9.4	7.5	13.2	0.55
	9	18	106.2	84.9	7.3	14.7	6.9	13.9	0.62
	8	16	193.9	86.2	6.0	29.8	3.1	15.4	0.75
Phe	9	18	41.6	83.2	3.0	5.5	7.2	13.2	0.51
	9	18	80.3	80.3	6.1	10.7	7.6	13.3	0.57
	8	16	203.9	81.6	9.5	22.5	4.7	11.0	0.54
Pyr	9	18	34.0	85.1	2.2	3.3	6.4	9.8	0.37
	8	16	63.2	84.3	2.2	5.3	3.5	8.4	0.35
	9	18	163.4	81.7	8.0	16.6	4.9	10.2	0.48

add 50 µL of the 1 µg/mL ^{13}C-PAHs solution and 850 µL isooctane. Cap the vial and vortex mix briefly.

F. Extraction and Cleanup Procedure

(*1*) Add 50 µL of the 1 µg/mL ^{13}C-PAHs solution to 10 ± 0.1 g of thoroughly homogenized seafood sample in a 50 mL polypropylene centrifuge tube.

(*2*) Vortex sample for 15 s and let equilibrate for 15 min.

(*3*) Add 5 mL (10 mL in the case of shrimp) of purified water and 10 mL ethyl acetate.

(*4*) Shake tube vigorously by hand for 1 min.

(*5*) Add 4 g of muffled anhydrous magnesium sulfate and 2 g sodium chloride, and seal the tube well (ensure that powder does not get into the screw threads or rim of the tube).

(*6*) Shake tube vigorously by hand for 1 min, ensuring that crystalline agglomerates are broken up sufficiently during shaking.

(*7*) Centrifuge tube at >1500 rcf for 10 min.

(*8*) Take a 5 mL aliquot of the upper ethyl acetate layer, add 50 µL isooctane as a keeper, and gently evaporate all ethyl acetate until only isooctane and co-extracted sample fat are left.

(*9*) Reconstitute in 1 mL hexane.

(*10*) Condition a silica SPE column (1 g silica gel with approximately 0.2 g of muffled anhydrous sodium sulfate on the top) with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane.

(*11*) Apply the extract in hexane onto the silica SPE cartridge.

(*12*) Elute with hexane–dichloromethane (3 + 1, v/v) using volume determined for the given silica gel SPE cartridges from the elution profiles of target analytes and fat, which are dependent on the silica deactivation, *see Note* (*4*) below. Collect the eluent.

(*13*) Add 0.5 mL isooctane (and 1-2 mL ethyl acetate) to the eluent as a keeper and gently evaporate down to 0.5 mL to remove hexane and dichloromethane from the final extract.

(*14*) Transfer the final extract into an autosampler vial for the GC/MS analysis.

Notes: (*1*) The fat capacity of the 1 g silica gel SPE column is approximately 0.1 g. If the 5 mL ethyl acetate extract aliquot contains more than 0.1 g fat, it is necessary to use a smaller aliquot volume to avoid sample breakthrough during the cleanup step.

(*2*) Ethyl acetate should not be present in the extract applied to the silica cartridge because it can affect the extract polarity, thus potentially retention of fat and analytes on the silica gel. The coextracted fat and 50 µL isooctane act as keepers during the first evaporation step (step *8*), thus the evaporation should be conducted gently until there is no significant change in the volume, i.e., until only the isooctane and coextracted fat are left in the evaporation tube or flask.

(*3*) Addition of 1-2 mL ethyl acetate to the eluent in step *13* is recommended for a better control of the evaporation process and higher absolute recoveries of volatile PAHs.

(*4*) The deactivation and storage of silica gel SPE cartridges can vary, potentially resulting in different amounts of water in the silica, thus its potentially different retention characteristics. Therefore, it is important to test the elution profiles of PAHs and fat and determine the optimum volume of the elution solvent to ensure adequate analyte recoveries and fat cleanup. The following procedure is recommended:

(**a**) Prepare a PAH solution in hexane by combining 50 µL of the Working PAH Solution A and 1 mL hexane in a vial. Mix well and apply onto a silica SPE column (1 g silica gel with approximately 0.2 g of muffled anhydrous sodium sulfate on the top), which was conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane.

(**b**) Elute with 10 mL hexane–dichloromethane (3 + 1, v/v),

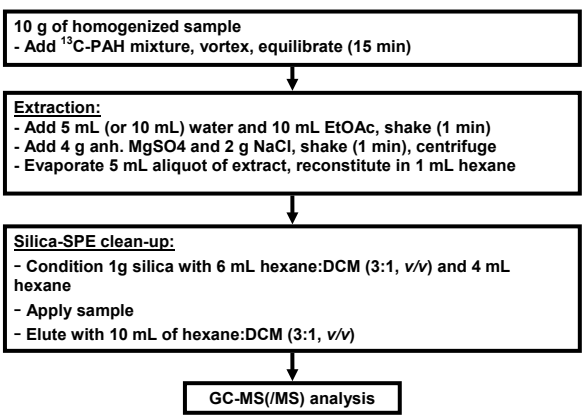

Figure 2014.08A. Flow chart of the method for determination of PAHs in seafood using GC/MS.

Table 2014.08E. Analyte concentrations in the mixed stock standard solution

Analyte	Concentration, µg/mL
Anthracene	10
Benz[*a*]anthracene	10
Benzo[*a*]pyrene	10
Benzo[*b*]fluoranthene	10
Benzo[*g,h,i*]perylene	10
Benzo[*k*]fluoranthene	10
Chrysene	25
Dibenz[*a,h*]anthracene	10
Fluoranthene	25
Fluorene	10
Indeno[1,2,3-*cd*]pyrene	10
Naphthalene	50
Phenanthrene	25
Pyrene	25
1-Methylnaphthalene	25
2,6-Dimethylnaphthalene	25
1-Methylphenanthrene	25
1,7-Dimethylphenanthrene	10
3-Methylchrysene	25

collecting 0.5 mL elution fractions in 20 evaporation tubes or flasks. Add 0.5 mL isooctane to each elution fraction and evaporate down to 0.5 mL using the optimized evaporation conditions. Analyze each fraction by GC/MS.

(**c**) Determine PAH elution profile by plotting analyte response (peak area or height) in a given fraction normalized to the sum of analyte responses in all tested fractions vs the elution volume. *See* Figure **2014.08B** for an example of a PAH elution profile. It is recommended to add an additional 0.5 mL on top of the determined elution fraction (corresponding to 100% recovery) as a safety margin ensuring good analyte recoveries in routine practice. This would result in the optimum elution volume of 7 mL for the silica cartridge tested in Figure **2014.08B**.

Table 2014.08F. Dilution scheme for preparation of the calibration standard solutions

Calibration level	Vol. of mixed stock standard solution, µL	Vol. of working PAH solution[a], µL	Vol. of working PAH solution B, µL	Vol. of ^{13}C-PAH 1 µg/mL solution, µL	Final vol.[a], µL
1	—	—	50	50	1000
2	—	—	100	50	1000
3	—	—	200	50	1000
4	—	—	500	50	1000
5	—	100	—	50	1000
6	—	200	—	50	1000
7	—	500	—	50	1000
8	100	—	—	50	1000

[a] Bring to volume using isooctane.

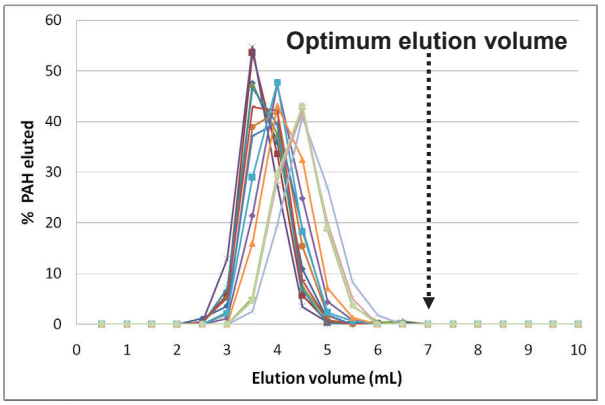

Figure 2014.08B. An example of elution profiles of PAHs on a silica gel SPE cartridge and determination of the optimum elution volume.

(**d**) To check the effectiveness of fat removal, dissolve 100 mg pure fish oil (or any suitable fat) in 1 mL hexane and apply it onto the silica gel cartridge, which was conditioned with 6 mL hexane–dichloromethane (3 + 1, v/v) and 4 mL hexane. Elute with the optimum elution volume of hexane–dichloromethane (3 + 1, v/v), which was determined in the previous step (e.g., 7 mL for the example in Figure **2014.08B**). Collect this fraction in an evaporation tube or flask, which empty weight (after heating in an oven to remove moisture) was recorded to 4 decimal places using an analytical balance. Elute the cartridge with additional 3 × 1 mL hexane–dichloromethane (3 + 1, v/v) and collect in three evaporation tubes/flasks of known empty weight.

(**e**) Evaporate the four elution fractions to dryness and gravimetrically determine the amount of fat eluting in each fraction by subtracting the empty weights from newly recorded weights after solvent evaporation. There should be no fat eluting in the optimum elution fraction for PAHs (this can also be observed visually in the tubes).

(**f**) If there is fat coeluting with PAHs, then the PAH and fat elution profiles have to be reexamined to determine optimum elution volume for PAH and fat separation (potentially sacrificing up to 5% of late-eluting PAH amounts if necessary) or a different silica gel cartridge has to be used.

G. GC/MS Analysis

(**a**) *GC conditions.*—Table **2014.08G** provides GC conditions that were used by the collaborative study participants. Other conditions (e.g., column, temperature and flow program, and injection technique and volume) can be used as long as the laboratory qualification criteria for separation, sensitivity, and linearity are met. The injection temperature or program needs to be optimized to enable quantitative transfer of less volatile PAHs. If programmable temperature vaporizer (PTV) solvent vent mode is used, solvent venting parameters (temperature, time, flow, pressure) need to be carefully optimized to prevent losses of the volatile PAHs, especially naphthalene. The separation criteria (demonstrated in Figure **2014.08C**) include (*1*) a baseline separation of benzo[*a*]pyrene and benzo[*e*]pyrene (concentration ratio of 1:5), (*2*) at least 50% valley separation of anthracene and phenanthrene (concentration ratio 1:2.5; evaluated for the anthracene peak), and (*3*) at least 50% valley

Table 2014.08G. GC conditions used by collaborative study participants

Laboratory No.	1	2	3	4	5
GC Instrument	Thermo Trace GC Ultra	Agilent 6890N	Agilent 6890N	Agilent 6890	Agilent 7890A
Inlet type	PTV (Thermo 816, RP - 2004)	PTV (Gerstel CIS 4)	S/SL (Agilent)	PTV (Gerstel CIS 4)	Multi-mode (Agilent)
Column type	Thermo TR-50MS	Restek Rxi-17Sil MS	Agilent J&W DB-17MS	Restek Rxi-17Sil MS	Restek Rxi-17Sil MS
Column dimension	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm
Retention gap dimension	NA	NA	NA	2 m × 0.25 mm id, 0.25 µm	NA
Run time, min	40	49.97	47.17	46	39.33
Oven temperature program	60°C (1 min), 12°C/min to 210°C, 8°C/min to 340°C (10 min)	50°C (4.3 min), 30°C/min to 220°C, 2°C/min to 240°C, 5°C/min to 360°C (6 min)	80°C (1 min), 30°C/min to 220°C, 2°C/min to 270°C (1.5 min), 5°C/min to 320°C	65°C (4 min), 30°C/min to 220°C (0.5 min), 3°C/min to 320°C (5 min)	65°C (0.5 min), 15°C/min to 220°C, 4°C/min to 330°C (1 min)
He flow/pressure program	1.2 mL/min (constant flow)	1.3 mL/min (constant flow)	1.3 mL/min (constant flow)	1.3 mL/min (15 min), 50 mL/min^2 to 2 mL/min	2 mL/min (constant flow)
Injection mode	PTV solvent split	PTV solvent vent	Pulsed splitless	PTV solvent vent (stop flow)	PTV solvent vent
Injection volume, µL	8	8	1	8	5
Inlet temperature (program)	50°C (2.3 min), 6.7°C/s to 300°C (1 min), 10°C/s to 350°C (10 min)	50°C (2.3 min), 12°C/s to 340°C	320°C	15°C (0.5 min), 12°C/min to 275°C	70°C (0.15 min), 600°C/min to 325°C (5 min)
Liner	Thermo, LVI Silcosteel, 2 mm id	Gerstel, CIS4 deactivated baffled liner, 2 mm id	Restek, single-gooseneck splitless, 4 mm id	Gerstel, CIS4 deactivated baffled liner, 2 mm id	Agilent, dimpled deactivated, 2 mm id
PTV solvent vent time	0.1 min	138 s	NA	0.5 min	0.1 min
PTV solvent vent flow, mL/min	50	50	NA	100	50
PTV solvent vent pressure, psi	NA	50	NA	1.80	5
Pressure pulse and duration	NA	NA	50 psi for 0.2 min	NA	NA
Split vent purge flow and start	50 mL/min, 1 min	20 mL/min	50 mL/min, 1 min	50 mL/min, 2.5 min	60 mL/min, 2.6 min
Gas saver flow and start time	15 mL/min, 3 min	NA	15 mL/min, 6 min	30 mL/min, 3 min	NA
MS transfer line temperature, °C	250	280	280	280	300
	6	7	8	9	10
GC instrument	Agilent 7890	Agilent 7890	Agilent 6890N	Agilent 6890N	Agilent 6890N
Inlet type	S/SL (Agilent)	S/SL (Agilent)	S/SL (Agilent)	PTV (Apex, ProSep 800 Plus)	PTV (Gerstel CIS 4)
Column type	Restek Rxi-17Sil MS	Restek Rxi-17Sil MS	Agilent J&W DB-EUPAH	Phenomenex ZB-50	Agilent J&W DB-EUPAH
Column dimension	30 m × 0.25 mm id, 0.25 µm	30 m × 0.25 mm id, 0.25 µm	20 m × 0.18 mm id, 0.14 µm	30 m × 0.25 mm id, 0.25 µm	20 m × 0.18 mm id, 0.14 µm
Retention gap dimension	1 m × 0.25 mm	NA	NA	NA	NA
GC run time, min	46.33	41.63	31.83	48.27	36.17
Oven temperature program	65°C (0.5 min), 15°C/min to 220°C, 4°C/min to 330°C (8 min)	80°C (4.3 min), 30°C/min to 220°C, 2°C/min to 240°C, 5°C/min to 300°C (1 min), 30°C/min to 350°C (8 min)	80°C (1 min), 30°C/min to 130°C, 12°C/min to 240°C, 3.5°C/min to 310°C	80°C (4.6 min), 30°C/min to 130°C (5 min), 10°C/min to 260°C (15 min), 10°C/min to 320°C (3 min); Post run: 80°C (1 min)	55°C (3 min), 30°C/min to 220°C, 2°C/min to 240°C, 3°C/min to 285°C; 15°C/min to 310°C (1 min); Post run: 330°C (2 min)

Table 2014.08G. (*continued*)

Laboratory No.	6	7	8	9	10
He flow/pressure program	1.5 mL/min (13 min), 10 mL/min^2 to 2 mL/min	1.5 mL/min (constant flow)	1 mL/min (constant flow)	1.3 mL/min (19.5 min), 50 mL/min^2 to 2 mL/min	2 mL/min (constant flow)
Injection mode	Hot splitless	Hot splitless	Pulsed splitless	Splitless	PTV solvent vent
Injection volume, µL	1	1	1	10	5
Inlet temperature (program)	300°C	280°C	320°C	60°C (2.25 min), 300°C/min to 335°C (45 min)	50°C (0.5 min), 600°C/min 400°C (15 min), 30°C/min 70°C
Liner	SGE, Focus liner	Agilent, single taper, glass wool-packed, 4 mm id	Agilent, double taper, 4 mm id	Apex, ProSep taper liner, 2 mm id	Agilent, PTV multi-baffle, 2 mm id
PTV solvent vent time	NA	NA	NA	NA	0.5 min
PTV solvent vent flow	NA	NA	NA	NA	100 mL/min
PTV solvent vent pressure	NA	NA	NA	NA	50 kPa
Pressure pulse and duration	NA	NA	50 psi for 0.2 min	NA	NA
Split vent purge flow and start	50 mL/min, 1 min	50 mL/min, 1 min	50 mL/min, 1 min	50.0 mL/min, 2.30 min	100 mL/min, 3 min
Gas saver flow and start time	20 mL/min, 3 min	20 mL/min, 2 min	15 mL/min, 6 min	NA	15 mL/min, 6 min
MS transfer line temperature, °C	300	280	320	300	325

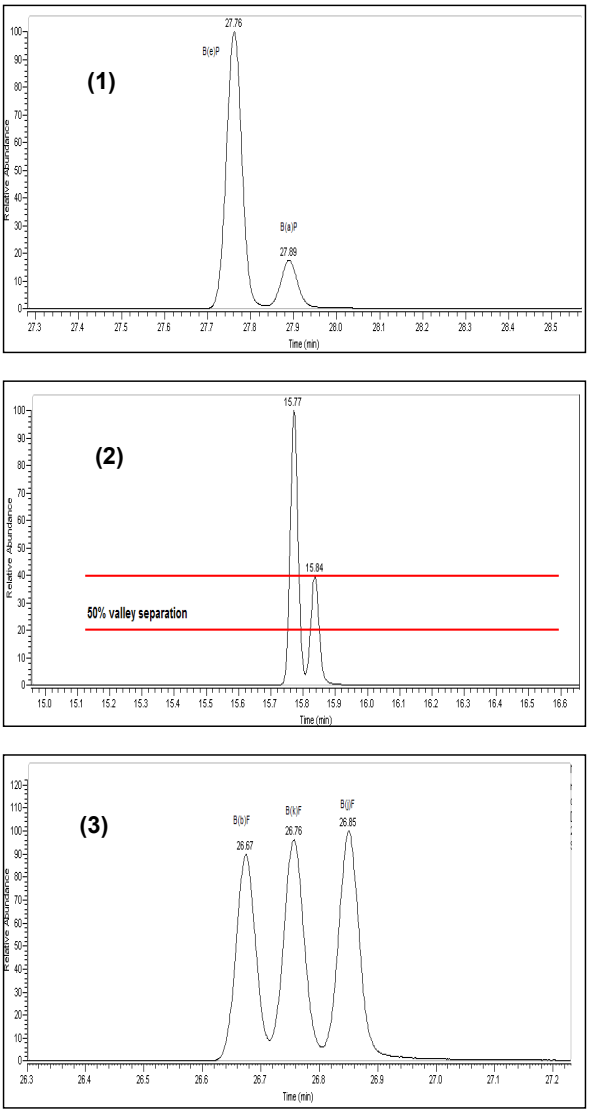

Figure 2014.08C. GC separation criteria: (1) A baseline separation of benzo[*a*]pyrene and benzo[*e*]pyrene (concentration ratio of 1:5), (2) at least 50% valley separation of phenanthrene and anthracene (concentration ratio 2.5:1; evaluated for the anthracene peak, which is the second peak in the figure), and (3) at least 50% valley separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*]fluoranthene (concentration ratio of 1:1:1).

separation for benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, and benzo[*k*]fluoranthene (concentration ratio of 1:1:1). *Note*: Criteria for separation of chrysene and triphenylene (another PAH critical pair) were not set for the collaborative study. For accurate quantitation of chrysene, at least 50% valley separation is recommended, which can be achieved using selective stationary phases.

The maximum oven temperature program may not exceed the maximum temperature limit for a given column. Backbone-modified columns, such as Rxi-17Sil MS or DB-17MS, are recommended for their better temperature stability and also good selectivity for critical PAH pairs or groups, including the anthracene/phenanthrene pair or benzofluoranthenes. Conduct proper inlet and column

Table 2014.08H. MS ions (*m/z*) and MS/MS precursor to product ion transitions used by study participants for quantification (quant) and identification (qual) of target PAHs and ^{13}C-PAHs using single-stage MS (single quadrupole and TOF) and tandem MS/MS (triple quadrupole) instruments, respectively

	Single quad/TOF MS		Triple quad MS/MS	
Compound	Quant	Qual	Quant	Qual
1,7-DMP	206	191	206>190	206>205, 206>165
1-MN	142	115	142>115	142>141, 142>116
1-MP	192	189	192>191	192>165
2,6-DMN	156	141, 144	156>115	156>141
3-MC	242	241	242>239	242>226
Ant	178	177	178>176	178>177, 178>151
BaA	228	226	228>226	228>224, 228>202
BaP	252	253	252>250	250>248, 252>224
BbF	252	253	252>250	250>248, 252>224
BghiP	276	277	276>274	274>272, 276>275
BkF	252	253	252>250	250>248, 252>224
Chr	228	226	228>226	228>224, 228>202
DBahA	278	276	278>276	276>274, 278>274
Fln	166	165	166>165	166>164, 166>163
Flt	202	200	202>200	202>201
IcdP	276	277	276>274	274>272, 276>248
Naph	128	127	128>102	128>127
Phe	178	177	178>176	178>177, 178>151
Pyr	202	200	202>200	202>201
13C-Ant	184	183	184>183	184>182, 184>156
13C-BaA	234	232	234>232	234>206
13C-BaP	256	257	256>254	256>228
13C-BbF	258	259	258>256	258>255
13C-BghiP	288	289	288>286	288>287
13C-BkF	258	259	258>256	258>255
13C-Chr	234	232	234>232	234>206
13C-DBahA	284	282	284>282	284>280
13C-Fln	172	171	172>171	172>170
13C-Flt	208	205	208>206	208>207
13C-IcdP	282	283	282>280	282>281
13C-Naph	134	133	134>133	134>105
13C-Phe	184	183	184>183	184>156
13C-Pyr	205	203, 206, 208	205>203	205>204

maintenance to ensure adequate operation of the GC instrument. Perform system checks.

(**b**) *MS conditions.*—Any GC/MS instrument (single quadrupole, triple quadrupole, TOF, or ion trap) with EI may be used as long as it provides results meeting the laboratory qualification requirements. The 10 study participants used the following instruments: single quadrupole (Agilent 5973–Laboratory 4; Agilent 5975B XL Inert–Laboratories 3 and 8–10; Agilent 5975C–Laboratories 6 and 7), triple quadrupole (Agilent 7000B–Laboratory 5; Thermo TSQ–Laboratory 1), and time-of-flight (Leco Pegasus 4D–Laboratory 2). Pay special attention to the optimization of the MS transfer line and MS source temperature. Higher MS source temperatures are recommended (e.g., ≥280°C for Agilent sources) to provide optimum analysis (quantitative transfers, minimum peak tailing) for less volatile PAHs.

Table **2014.08H** provides MS ions (*m/z*) and MS/MS transitions used by the study participants for quantification and identification of target PAHs and ^{13}C-PAHs using single-stage MS (single quadrupole and TOF) and tandem MS/MS (triple quadrupole) instruments, respectively.

Use adequate data acquisition rate (dwell times in scanning instruments) and solvent delay time. Perform air/water checks and autotune to verify and obtain adequate operation

Table 2014.08I. PAH analytes and corresponding ^{13}C-PAHs used for PAH signal normalization

Analyte	^{13}C-PAH used for signal normalization
Anthracene	Anthracene ($^{13}C_6$)
Benz[*a*]anthracene	Benz[*a*]anthracene ($^{13}C_6$)
Benzo[*a*]pyrene	Benzo[*a*]pyrene ($^{13}C_4$)
Benzo[*b*]fluoranthene	Benzo[*b*]fluoranthene ($^{13}C_6$)
Benzo[*g,h,i*]perylene	Benzo[*g,h,i*]perylene ($^{13}C_{12}$)
Benzo[*k*]fluoranthene	Benzo[*k*]fluoranthene ($^{13}C_6$)
Chrysene	Chrysene ($^{13}C_6$)
Dibenz[*a,h*]anthracene	Dibenz[*a,h*]anthracene ($^{13}C_6$)
Fluoranthene	Fluoranthene ($^{13}C_6$)
Fluorene	Fluorene ($^{13}C_6$)
Indeno[1,2,3-*cd*]pyrene	Indeno[1,2,3-*cd*]pyrene ($^{13}C_6$)
Naphthalene	Naphthalene ($^{13}C_6$)
Phenanthrene	Phenanthrene ($^{13}C_6$)
Pyrene	Pyrene ($^{13}C_6$)
1-Methylnaphthalene	Naphthalene ($^{13}C_6$)
2,6-Dimethylnaphthalene	Phenanthrene ($^{13}C_6$)
1-Methylphenanthrene	Phenanthrene ($^{13}C_6$)
1,7-Dimethylphenanthrene	Phenanthrene ($^{13}C_6$)
3-Methylchrysene	Chrysene ($^{13}C_6$)

of the instrument. Verify identification of the analyte peaks by comparing the ion ratios of contemporaneously analyzed calibration standards, which have been analyzed under the same conditions.

(**c**) *Injection sequence.*—Bracket the seven test samples with two sets of calibration standards. Inject solvent blanks after the calibration level 8 (highest) standard and after the samples. In addition, analyze a reagent blank with each set of samples. Inject only once from each vial, thus preventing potential losses of volatile PAHs and/or contamination.

H. Calculations

Quantification is based on linear least-squares calibration of analyte signals (S_{PAH}) divided by signals ($S_{13C\text{-}PAH}$) of corresponding ^{13}C-labeled internal standards (*see* Table **2014.08I**) plotted versus analyte concentrations. Peak areas are generally preferred as signals used for the quantification, but peak heights should be used for peaks that are not well resolved, such as in the case of anthracene and phenanthrene. The analyte concentrations in the final extract (c_{PAH}, µg/L) are determined from the equation:

$$c_{PAH} = [(S_{PAH}/S_{13C\text{-}PAH}) - b]/a$$

where a is the slope of the calibration curve and b is the y-intercept.

The concentration of PAHs in the sample (C, µg/kg) is then calculated:

$$C = (c_{PAH}/c_{13C\text{-}PAH}) \times (X_{13C\text{-}PAH}/m)$$

where $c_{13C\text{-}PAH}$ is the concentration of the corresponding ^{13}C-PAH in the calibration standard solutions (in µg/L); $X_{13C\text{-}PAH}$ is the amount of the corresponding ^{13}C-PAH added to the sample (in ng); and m is the sample weight (in g). Based on the method procedure and preparation of the calibration standard solutions, $c_{13C\text{-}PAH}$ is 50 µg/L, $X_{13C\text{-}PAH}$ is 50 ng, and m for the test samples is 10 g.

In the collaborative study, eight concentration levels were used for the calibration, corresponding to 5, 10, 20, 50, 100, 200, 500, and 1000 µg/L for benzo[*a*]pyrene and other lower-level PAHs, to 12.5, 25, 50, 125, 250, 500, 1250, and 2500 µg/L for higher-level PAHs, except for naphthalene that was present at 25, 50, 100, 250, 500, 1000, 2500, and 5000 µg/L. Coefficients of determination (r^2) should be 0.990 or greater and back-calculated concentrations of the calibration standards should not exceed ±20% of theoretical. For lower concentration levels, a limited calibration curve (without the higher-end concentration points) may be used for better accuracy. If a well-characterized quadratic relationship occurs, then a best-fitted quadratic curve may be used for calibration. Otherwise, if the back-calculated concentrations exceed ±20% of theoretical, normalized signals of the nearest two calibration standards that enclose the analyte signal in the sample can be used to interpolate the analyte concentration in the final extract.

Results and Discussion

Laboratory Qualification Phase

The analysis of PAHs poses several difficulties due to their physicochemical properties and occurrence in the environment and various materials that can lead to contamination issues. PAH properties, such as their volatility, polarity, and structure, affect their GC separation, MS determination/identification, and recoveries during solvent evaporation and silica SPE steps. To allow for flexibility and the use of various instruments, equipment, and columns, the Study Directors did not want to prescribe the use of a specific GC/MS instrument, GC column and separation conditions, silica SPE cartridge, and evaporation technique, equipment, or conditions. For this reason, they developed performance-based criteria for the GC/MS analysis (including separation of critical PAH pairs/groups, calibration range, or carryover), optimum elution volume in the SPE step (based on the elution profiles of PAHs and fat dependent on the silica deactivation), and evaporation conditions (to avoid significant loses of volatile PAHs, mainly naphthalene). These criteria were part of the laboratory qualification phase to help laboratories optimize conditions independent of their instrument/equipment choice or availability. This was also a very important consideration for the future implementation of the method in other laboratories.

Another essential step in the laboratory qualification phase involved check of reagent blanks for potential PAH contamination. The concentrations of all analytes in the reagent blanks had to be below the concentrations in the lowest calibration level standard. For naphthalene, levels below the second lowest calibration standard (equivalent to 5 ng/g of naphthalene in the sample) were still acceptable if the source of contamination could not be eliminated, such as by selection of a silica gel SPE column from a different vendor (or preparation of silica gel columns in-house), heating of glassware, addition of a hydrocarbon trap to the nitrogen lines used for solvent evaporation, etc. Some laboratories found that their reagent

Table 3. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the low PAH level in shrimp

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC S10	17.4	17.9	24.9	14.1	21.4	21.2	18.5	23.4	26.4
	SFC S11	23.2	25.6	27.0	14.2	21.4	20.8	23.1	23.1	27.4
1-MN	SFC S10	44.8	33.3	23.5	17.9	23.6	22.1	17.8	18.6	20.5
	SFC S11	24.6	18.0	25.4	19.6	26.4	20.2	22.3	19.3	17.5
1-MP	SFC S10	9.1	8.4	10.4	7.5	9.9	10.7	8.8	9.8	11.5
	SFC S11	10.7	10.5	12.3	7.5	9.8	10.4	11.2	9.3	11.9
2,6-DMN	SFC S10	32.0[a]	16.8	17.4	21.4	14.8	13.7	13.1	13.1	14.8
	SFC S11	35.3[a]	15.4	16.7	20.1	14.2	14.0	16.7	13.0	13.9
6-MC	SFC S10	7.8	11.6	12.5	10.9	10.6	10.8	9.7	11.9	11.8
	SFC S11	8.4	10.8	13.6	11.0	10.6	10.7	11.9	12.4	11.9
Ant	SFC S10	3.8	5.2	5.6	4.7	4.7	5.1	4.0	5.0	5.2
	SFC S11	4.6	5.2	5.9	4.7	4.7	5.0	5.1	5.1	5.0
BaA	SFC S10	3.9	5.5	4.6	4.7	4.6	4.7	4.2	5.1	4.9
	SFC S11	4.1	5.2	5.5	4.7	4.6	4.6	5.2	5.2	4.9
BaP	SFC S10	1.4	2.2	2.0	1.9	1.8	1.9	1.7	2.2	1.9
	SFC S11	1.6	2.0	2.2	1.9	1.8	1.9	2.1	2.2	1.9
BbF	SFC S10	3.7	5.2	5.1	4.8	4.6	4.7	4.3	5.1	4.7
	SFC S11	4.3	5.4	5.8	4.8	4.5	4.7	5.2	5.1	5.0
BghiP	SFC S10	1.4	2.2	1.9	1.9	1.8	1.9	1.7	2.0	2.7[a]
	SFC S11	1.6	2.1	2.2	1.9	1.8	1.9	2.0	2.0	3.5[a]
BkF	SFC S10	1.4	2.3	2.2	2.0	1.9	1.7	1.8	2.2	2.0
	SFC S11	1.6	2.2	2.3	2.0	1.9	1.6	2.3	2.2	2.0
Chr	SFC S10	12.3	17.6	15.5	14.9	14.2	14.7	13.2[a]	15.7	15.9
	SFC S11	13.1	16.8	16.9	15.0	14.3	14.5	16.2[a]	16.3	16.0
DBahA	SFC S10	1.3	2.1	2.2	2.0	1.8	2.0	1.7	2.1	2.3
	SFC S11	1.6	2.0	1.9	1.9	1.7	1.9	2.2	2.2	1.6
Fln	SFC S10	4.3	5.3	5.5	4.9	4.8	5.0	4.2[a]	5.6	6.0
	SFC S11	4.3	5.4	5.6	4.9	4.8	5.0	5.2[a]	5.4	5.9
Flt	SFC S10	7.2	10.6	9.8	9.6	9.2	9.5	8.5	10.6	10.8
	SFC S11	8.2	10.7	10.7	9.7	9.2	9.5	10.4	10.6	10.1
IcdP	SFC S10	1.4	2.2	1.9	2.0	1.7	2.0	1.7	2.3	2.1
	SFC S11	1.6	2.1	2.0	2.0	1.7	2.0	2.1	2.3	2.1
Naph	SFC S10	35.8	29.6	28.9	26.0	27.3	26.1	23.3	28.1	33.9[a]
	SFC S11	27.1	26.2	27.7	26.3	27.1	24.7	29.5	29.0	42.5[a]
Phe	SFC S10	12.2	16.1	14.4	14.7	15.0	14.9	12.7[b]	15.6	16.2
	SFC S11	13.2	16.3	16.1	14.6	14.9	15.2	15.9[b]	15.5	16.4
Pyr	SFC S10	12.0	16.0	14.4	14.8	14.2	14.6	12.6	15.6	16.0
	SFC S11	12.8	15.8	16.6	14.9	14.2	14.4	15.7	15.7	15.7

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 4. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the mid PAH level in shrimp

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC S9	28.6	17.4	26.2	14.9	21.6	21.0	20.3	23.9	25.1
	SFC S14	27.9	23.8	28.4	14.9	23.3	21.1	22.4	21.8	26.6
1-MN	SFC S9	84.6	111.5	89.1	59.8	83.7	70.1	72.8	75.4	56.9
	SFC S14	99.7	101.4	101.7	66.1	85.9	74.9	81.9	83.3	67.6
1-MP	SFC S9	26.8	17.8	29.6	18.6	24.5	24.7	23.7	26.1	27.1
	SFC S14	26.6	23.9	30.8	18.5	25.6	25.0	26.5	29.2	24.2
2,6-DMN	SFC S9	80.0[a]	46.5	40.8	40.3	38.5	33.4	38.1	31.3	23.4
	SFC S14	102.1[a]	43.2	42.9	51.7	37.8	31.7	42.3	27.0	36.8
6-MC	SFC S9	27.0	33.9	37.2	30.0	30.9	30.5	30.7	37.5	33.6
	SFC S14	24.5	33.0	38.7	30.2	31.3	30.8	33.6	32.0	33.2
Ant	SFC S9	9.2	10.8	13.4	9.4	9.8	9.8	9.3	11.1	11.8
	SFC S14	8.3	10.4	15.2	9.5	9.8	9.7	10.8	12.2	10.0
BaA	SFC S9	13.3	17.0	16.2	14.3	14.4	14.0	14.1	17.0	15.4
	SFC S14	12.4	16.2	15.5	14.3	14.6	14.2	15.6	15.2	15.9
BaP	SFC S9	4.5	5.7	5.1	4.9	4.7	4.7	4.7	4.5	5.5
	SFC S14	3.9	5.2	5.9	4.9	4.7	4.7	5.1	5.3	5.0
BbF	SFC S9	8.8	10.8	10.6	9.8	9.3	9.2	9.4	10.2	10.1
	SFC S14	8.8	10.5	11.1	9.7	9.4	9.4	10.1	10.0	9.5
BghiP	SFC S9	4.6	5.6	5.8	4.9	4.6	4.7	4.6	4.5	11.5[b]
	SFC S14	4.3	5.3	5.9	4.8	4.6	4.8	4.9	5.1	2.5[b]
BkF	SFC S9	7.9	8.6	9.5	7.7	7.7	8.0	7.6	7.6	8.5
	SFC S14	6.5	8.6	9.6	8.0	7.7	8.1	8.3	8.2	8.3
Chr	SFC S9	46.8	52.4	55.8	48.4	48.2	47.7	47.8	57.6	54.7
	SFC S14	42.8	50.2	57.8	48.5	49.1	48.1	52.3	51.4	53.1
DBahA	SFC S9	4.2	6.1	5.1	5.0	4.8	4.9	4.7	6.0	4.3
	SFC S14	4.0	5.3	5.7	5.0	4.8	5.0	5.2	5.4	4.9
Fln	SFC S9	14.8	15.8	16.6	14.4	14.6	14.8	14.0	16.9	17.0
	SFC S14	13.3	15.7	17.2	14.6	14.7	14.4	15.5	15.4	16.4
Flt	SFC S9	21.7	26.6	27.5	24.6	23.8	23.5	23.4	26.7	29.1
	SFC S14	19.9	26.7	28.9	24.6	24.0	23.2	26.7	25.6	25.0
IcdP	SFC S9	4.4	5.7	4.5	5.0	4.8	5.3	4.8	5.1	6.7
	SFC S14	4.1	5.4	5.5	5.0	4.8	5.1	5.2	5.4	5.1
Naph	SFC S9	86.2	88.1	86.8	75.9	80.2	71.9	79.4	75.3	90.1
	SFC S14	104.3	86.4	90.6	73.6	80.6	76.2	90.2	80.2	97.8
Phe	SFC S9	46.8	50.9	54.2	47.6	48.9	47.9	46.5	48.7	51.3
	SFC S14	44.7	50.3	56.2	47.8	50.0	47.4	51.3	50.7	53.5
Pyr	SFC S9	36.5	41.8	45.3	39.3	39.2	38.6	38.2	38.8	43.6
	SFC S14	32.6	42.0	46.6	39.4	39.7	38.5	42.3	41.5	42.1

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 5. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the high PAH level in shrimp

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC S8	26.5	19.3	21.1	16.2	20.0	19.9	16.1	23.0	28.2
	SFC S13	32.4	17.9	22.2	16.9	21.2	20.7	20.4	21.7	26.2
1-MN	SFC S8	318.7	195.9	243.8	145.0	202.9	197.9	146.0	236.8	105.8
	SFC S13	307.7	180.7	241.9	179.9	213.2	208.7	177.5	205.6	149.7
1-MP	SFC S8	122.8	103.8	122.0	102.3	120.8	115.2	92.3	125.8	150.3
	SFC S13	123.3	104.4	124.5	101.4	121.5	118.7	114.6	141.7	143.1
2,6-DMN	SFC S8	311.3[a]	148.1	167.6	128.2[a]	148.2	154.2	128.7	134.4	153.8
	SFC S13	706.5[a]	101.8	183.3	275.1[a]	166.9	144.4	155.3	130.4	136.0
6-MC	SFC S8	127.0	162.0	154.6	140.1	141.3	138.3	115.6	145.4	164.3
	SFC S13	126.7	149.8	152.3	151.5	142.6	143.9	137.8	166.5	153.4
Ant	SFC S8	35.8	42.4	47.1	37.6	37.7	36.7	28.6	40.6	44.6
	SFC S13	35.0	39.4	40.6	36.1	37.8	37.6	34.9	44.0	44.4
BaA	SFC S8	54.8	65.7	57.9	55.0	55.4	53.1	44.3	58.6	64.7
	SFC S13	52.5	63.3	55.5	54.0	55.0	55.1	53.1	60.2	61.0
BaP	SFC S8	22.9	25.1	25.0	22.9	22.8	22.1	18.8	23.9	26.0[a]
	SFC S13	21.9	24.3	23.9	22.0	22.7	23.2	22.3	25.3	37.8[a]
BbF	SFC S8	67.6	83.4	74.2	70.0	69.6	68.8	57.0	72.1	82.9
	SFC S13	70.4	73.4	69.6	67.6	69.0	70.5	67.4	76.5	79.1
BghiP	SFC S8	17.2	20.5	19.7	17.8	17.5	17.5	14.4	18.6	52.0[a]
	SFC S13	18.0	18.3	18.8	17.4	17.4	18.3	17.2	19.9	15.8[a]
BkF	SFC S8	37.4	41.9	41.4	35.9	38.2	37.3	30.7	38.5	41.4
	SFC S13	36.3	39.4	39.6	37.3	37.2	39.1	36.3	41.6	40.1
Chr	SFC S8	160.8	186.8	174.4	164.4	164.0	159.7	131.8	167.6	194.0
	SFC S13	155.1	175.0	165.3	161.1	163.6	164.4	157.3	186.2	181.5
DBahA	SFC S8	12.8	16.2	14.5	14.3	13.5	13.0	11.3	14.3	13.6
	SFC S13	12.4	14.4	13.3	13.4	13.4	13.4	13.6	15.5	15.1
Fln	SFC S8	48.5	53.2	48.6	46.2	46.5	46.2	35.6	47.8	52.7
	SFC S13	45.7	50.9	45.8	46.5	46.3	46.8	42.9	49.9	52.0
Flt	SFC S8	86.3	103.6	96.7	92.8	92.1	87.9	72.1	96.0	111.4
	SFC S13	89.6	96.8	96.0	92.2	92.6	90.8	87.4	103.2	102.9
IcdP	SFC S8	17.3	20.9	18.6	18.2	17.5	17.6	14.4	19.1	22.9
	SFC S13	17.0	19.7	17.3	17.7	18.0	17.9	16.9	20.5	20.1
Naph	SFC S8	244.7	137.6	174.1	143.1	147.9	147.2	112.0	166.8	260.0[a]
	SFC S13	218.7	129.4	166.1	141.6	149.4	148.9	137.6	173.7	184.9[a]
Phe	SFC S8	155.7	186.0	166.5	165.2	165.1	162.3	126.0	171.2	201.3
	SFC S13	153.8	176.9	173.8	164.2	169.0	165.8	153.7	183.0	185.2
Pyr	SFC S8	109.3	125.9	122.7	117.6	115.6	113.6	91.3[a]	119.9	123.2
	SFC S13	106.4	123.6	118.3	116.3	115.3	116.4	110.4[a]	128.6	127.4

[a] Outliers removed by Cochran test.

Table 6. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the low PAH level in mussel

		Laboratory No.									
PAH	Sample ID	1	2	3	4	5	6	7	8	9	10
1,7-DMP	SFC M1	40.8	41.5	44.4	23.9	45.4	39.2	40.9	44.5	30.9	37.9
	SFC M6	18.2	47.1	51.3	25.9	44.4	38.9	43.2	43.8	29.0	31.6
1-MN	SFC M1	17.0	21.2	23.2	10.4	19.1	17.5	22.1	24.6	16.2	16.0
	SFC M6	27.9	16.5	27.6	12.6	18.9	18.8	21.2	20.2	16.8	18.6
1-MP	SFC M1	33.7[a]	8.3	9.9	5.7	10.4	9.6	10.4	10.8	9.5	13.1
	SFC M6	18.0[a]	8.0	10.7	6.6	10.1	10.1	10.9	11.0	10.6	10.8
2,6-DMN	SFC M1	21.8	9.5	13.8	12.3	14.8	11.7	13.4	14.5	11.8	12.0
	SFC M6	17.8	10.8	14.6	16.5	14.8	12.4	11.9	15.7	11.0	14.6
3-MC	SFC M1	6.4	10.4	10.5	8.0	10.2	10.6	11.0	10.9	8.9	11.4
	SFC M6	6.1	8.1	11.3	10.2	10.0	10.8	11.1	10.8	8.8	11.8
Ant	SFC M1	4.9	4.4	3.1	1.2	2.3	5.0	4.7	4.9	3.8	4.9
	SFC M6	4.6	3.6	3.6	1.5	2.3	5.0	4.8	5.2	3.6	4.5
BaA	SFC M1	3.9	4.7	4.0	2.5	4.1	4.6	4.6	4.7	3.9	5.2
	SFC M6	4.2	4.0	4.9	3.1	4.1	4.5	4.7	4.5	3.8	5.1
BaP	SFC M1	1.3	2.1	1.8	1.0	1.3	0.1[b]	1.8	1.9	1.5	1.6
	SFC M6	1.4	2.0	1.9	1.2	1.2	0.1[b]	1.8	1.8	1.3	1.6
BbF	SFC M1	4.4	5.3	4.8	3.9	4.8	4.9	4.8	4.8	4.0	5.6
	SFC M6	4.7	4.3	5.1	4.7	4.7	4.3	4.8	4.7	4.1	5.7
BghiP	SFC M1	1.8	2.3[a]	2.0	1.7	2.0	2.0	2.0	2.1	1.7	2.3
	SFC M6	1.7	1.7[a]	2.2	2.0	1.9	2.0	2.2	1.9	1.7	2.3
BkF	SFC M1	1.8	1.8	2.2	1.6	2.0	1.8	1.9	2.1	47.0[a]	2.5
	SFC M6	1.8	1.9	2.1	2.0	1.9	2.0	1.8	2.0	1.6[a]	2.1
Chr	SFC M1	12.7	14.5	14.3	11.7	14.6	14.4	14.3	14.9	12.9	16.9
	SFC M6	13.2	12.3	15.3	14.2	14.3	14.4	14.4	14.0	12.9	17.0
DBahA	SFC M1	1.7	2.2	2.0	1.5	1.8	1.9	1.9	1.9	2.1	2.2
	SFC M6	1.7	1.7	1.9	1.7	1.8	1.8	1.9	1.8	1.6	2.2
Fln	SFC M1	5.3	6.2	5.6	5.5	5.1	5.1	5.0	5.1	4.5	6.2
	SFC M6	5.2	6.4	5.7	6.1	5.0	5.3	4.8	5.2	4.6	5.9
Flt	SFC M1	8.9	10.1	9.9	19.5[b]	10.0	12.4	9.6	10.2	10.8	12.0
	SFC M6	11.0	8.1	10.9	20.7[b]	9.7	12.5	9.8	9.8	7.5	11.2
IcdP	SFC M1	1.8	2.4	1.9	1.6	2.0	1.9	1.9	2.0	1.9	2.3
	SFC M6	1.6	1.9	2.1	1.9	2.0	2.0	2.0	1.9	1.7	2.4
Naph	SFC M1	20.3[a]	27.6	26.7	20.2	23.1	20.2	26.2	26.4	23.8	14.8
	SFC M6	36.0[a]	28.9	30.6	18.7	23.3	20.9	26.5	23.2	23.9	20.8
Phe	SFC M1	15.8	27.5[b]	14.2	12.7	15.2	14.2	14.5	15.3	14.1	21.2[b]
	SFC M6	13.9	26.7[b]	15.6	14.3	14.9	14.5	14.8	15.4	12.9	17.5[b]
Pyr	SFC M1	14.9	14.4	14.7	11.8	14.5	13.7	14.1	14.7	13.0	16.8
	SFC M6	14.2	11.9	15.4	13.2	14.2	14.0	14.4	14.0	12.9	16.8

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 7. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the mid PAH level in mussel

		Laboratory No.									
PAH	Sample ID	1	2	3	4	5	6	7	8	9	10
1,7-DMP	SFC M3	5.7[a]	48.0	45.9	27.4	46.1	40.5	42.9	43.8	28.2	36.6
	SFC M5	39.0[a]	38.5	41.7	31.0	44.1	38.9	42.1	57.2	28.8	28.1
1-MN	SFC M3	79.1	92.7	136.2	97.2	95.5	90.3	105.4	110.0	103.5	72.1
	SFC M5	78.2	105.0	117.3	87.2	97.2	103.0	109.8	119.5	87.4	50.7
1-MP	SFC M3	60.2[a]	55.2	54.3	32.2	52.2	47.1	50.6	50.5	31.5	50.9
	SFC M5	870.8[a]	41.7	48.0	30.1	49.1	44.9	51.0	63.2	31.7	40.3
2,6-DMN	SFC M3	21.5[a]	64.6	69.5	92.3	79.9	63.1	57.6	62.3	43.0	71.7[a]
	SFC M5	59.2[a]	63.6	62.1	85.7	74.9	59.4	61.7	64.0	43.3	92.5[a]
3-MC	SFC M3	70.3	81.5	101.6	80.7	93.8	93.4	93.3	91.4	78.6	98.9
	SFC M5	69.8	86.6	95.8	70.4	87.4	90.3	95.4	97.3	69.5	96.9
Ant	SFC M3	22.5	17.5	16.5	7.1	8.2	16.5	14.5	13.8	12.3	13.5
	SFC M5	14.9	13.6	14.4	11.0	6.6	13.7	14.8	15.2	10.8	14.7
BaA	SFC M3	22.3	21.0	24.8	18.4	21.5	23.2	22.7	22.3	19.6	23.9
	SFC M5	22.2	21.3	23.1	16.3	18.8	21.7	23.3	22.3	17.1	23.5
BaP	SFC M3	7.4	9.1	10.0	6.6	6.8	1.1[b]	9.2	9.8	7.7	8.4
	SFC M5	7.6	8.5	8.8	5.6	5.9	1.1[b]	9.4	9.9	6.4	7.8
BbF	SFC M3	28.2	27.3	29.2	24.0	28.9	31.6	27.9	27.6	24.3	29.9
	SFC M5	28.3	27.3	27.1	21.0	26.9	25.0	28.4	26.9	21.3	29.8
BghiP	SFC M3	8.8	9.1	10.8	8.1	9.5	9.8	10.0	9.7	7.5	11.0
	SFC M5	9.5	9.0	9.8	7.1	8.9	9.3	10.1	9.3	7.3	10.8
BkF	SFC M3	18.7	18.1	22.0	15.2	19.5	21.0	19.3	19.2	16.1	21.9
	SFC M5	18.8	18.3	20.0	14.0	18.2	17.2	19.0	18.9	13.9	21.4
Chr	SFC M3	92.7	86.1	100.8	79.7	98.0	95.2	93.2	92.0	86.0	105.2
	SFC M5	94.5	91.3	94.0	70.8	91.4	91.7	94.9	95.5	76.0	102.0
DBahA	SFC M3	8.8	9.4	9.7	8.0	9.4	9.9	9.6	9.5	6.3	11.0
	SFC M5	8.5	9.2	8.9	7.1	8.8	8.9	10.0	9.5	7.5	11.2
Fln	SFC M3	24.8	25.0	26.8	29.2	25.2	24.9	24.5	24.5	23.9	28.5
	SFC M5	24.3	25.6	25.1	29.6	23.6	24.1	25.1	26.0	21.9	26.5
Flt	SFC M3	47.8	45.5	49.7	46.6	50.8	55.3	48.1	48.2	39.2	55.6
	SFC M5	53.7	47.6	46.8	42.1	47.2	54.5	49.0	49.5	46.6	53.3
IcdP	SFC M3	9.0	9.6	10.1	8.1	9.7	10.1	9.5	9.7	8.6	11.3
	SFC M5	9.5	9.3	9.8	7.2	9.1	9.2	9.9	9.5	6.9	11.4
Naph	SFC M3	91.8	125.4	134.8	84.0	104.1	97.1	124.2	129.6	123.9	71.7
	SFC M5	84.6	122.2	127.8	65.1	107.7	114.8	124.8	135.3	109.7	40.0
Phe	SFC M3	92.1	206.3[b]	102.0	81.9	102.1	93.7	95.1	94.3[a]	84.3	98.8
	SFC M5	97.1	191.3[b]	93.9	76.3	96.2	90.5	96.6	119.2[a]	82.5	107.0
Pyr	SFC M3	79.2	71.9	80.3	62.0	75.6	71.2	72.4	72.2	64.2	80.2
	SFC M5	75.4	74.6	75.8	55.5	70.4	69.3	73.3	72.0	58.2	76.7

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 8. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the high PAH level in mussel

		Laboratory No.									
PAH	Sample ID	1	2	3	4	5	6	7	8	9	10
1,7-DMP	SFC M2	34.5	39.8	41.4	26.1	44.7	40.4	46.8	43.6	30.2	37.6
	SFC M7	22.4	34.0	42.2	26.5	47.0	39.3	48.5	56.7	30.7	45.8
1-MN	SFC M2	183.7	222.9[a]	211.8	199.1	193.9	195.7	196.9	190.0	206.8	387.8[b]
	SFC M7	274.7	459.3[a]	218.8	241.5	198.7	208.8	199.6	220.5	199.4	62.0[b]
1-MP	SFC M2	431.7[b]	113.4	122.8	81.6	128.5	115.8	135.8	123.1	79.6	125.9
	SFC M7	1752.2[b]	102.7	129.2	81.9	133.2	112.6	137.7	146.5	79.7	159.3
2,6-DMN	SFC M2	139.5	128.0	138.5	214.2	177.8	143.4	142.1	161.2	98.7	184.6
	SFC M7	121.6	135.2	158.1	244.0	187.9	141.7	149.4	144.7	94.2	171.3
3-MC	SFC M2	100.6	133.6	147.7	127.2	146.5	146.7	150.4	138.0	127.4	149.4
	SFC M7	103.5	130.3	156.7	128.4	151.9	144.9	151.9	145.6	121.6	157.7
Ant	SFC M2	35.3	44.7[b]	39.9	19.9	20.9	37.7	37.1	33.2	31.2	29.0
	SFC M7	31.7	33.9[b]	42.8	16.3	20.3	38.9	37.5	33.7	31.7	29.9
BaA	SFC M2	51.1	49.3	52.2	43.4[b]	49.9	53.3	54.9	50.0	47.7[a]	54.1
	SFC M7	52.0	50.7	55.0	41.7[b]	51.2	52.9	55.8	53.3	476.3[b]	56.1
BaP	SFC M2	19.1	19.8	22.3	15.6	16.6	3.0[a]	22.4	22.4	19.8	17.6
	SFC M7	18.3	19.4	22.9	15.1	17.0	3.0[a]	22.6	20.8	18.5	17.5
BbF	SFC M2	74.8	68.2	65.5	59.0	71.1	74.8	70.4	66.6	62.7	71.9
	SFC M7	71.8	67.2	68.5	58.8	74.1	77.2	71.2	69.5	60.3	78.1
BghiP	SFC M2	18.2	18.0	18.2	15.1	17.9	18.3	18.9	17.4	16.3	19.5
	SFC M7	18.8	17.6	19.7	14.9	18.7	18.0	19.3	18.4	15.2	20.3
BkF	SFC M2	38.8	33.8	38.8	30.8	37.3	36.3	38.3	36.9	30.5	41.7
	SFC M7	37.9	35.0	37.8	30.6	37.9	39.5	38.2	37.7	30.4	43.8
Chr	SFC M2	159.8	158.5	153.2	137.7	166.6	163.9	164.1	152.1	150.9	174.8
	SFC M7	166.8	152.4	165.9	139.2	172.3	161.0	165.6	161.0	145.3	184.1
DBahA	SFC M2	13.7	13.7	13.6	12.1	13.7	13.3	14.8	13.2	13.2	15.0
	SFC M7	13.9	13.0	14.5	10.6	14.3	13.2	14.8	14.0	12.3	15.4
Fln	SFC M2	50.2	49.0	45.8	59.2[a]	47.5	47.0	47.3	46.0	49.4	50.7
	SFC M7	49.8	49.1	49.8	59.4[a]	48.0	46.6	48.4	43.0	45.1	53.0
Flt	SFC M2	92.6	88.7	86.7	66.5[a]	96.2	96.5	93.4	89.7	93.1	100.2
	SFC M7	104.7	88.4	93.6	63.4[a]	98.1	93.7	94.7	84.1	79.6	104.3
IcdP	SFC M2	17.8	18.3	17.2	15.5	18.7	18.6	18.4	17.3	16.7	19.0
	SFC M7	17.0	16.6	18.7	15.3	19.7	19.3	18.5	18.3	15.2	21.3
Naph	SFC M2	125.2[b]	149.8	143.7	108.7	134.0	138.5	159.5	165.5	164.9	246.4[b]
	SFC M7	197.9[b]	160.7	160.0	104.6	141.0	144.4	159.0	165.6	146.5	34.3[b]
Phe	SFC M2	159.7	452.2[b]	159.4	142.5	173.2	161.0	166.2	162.4	143.6	164.0[b]
	SFC M7	178.0	366.3[b]	170.3	142.9	177.6	159.0	169.8	167.6	139.0	200.9[b]
Pyr	SFC M2	133.5	119.2	114.3	102.2	119.8	114.8	118.6	108.3	104.4	124.3
	SFC M7	123.0	117.5	122.6	102.9	125.1	113.3	120.1	119.2	97.4	128.6

[a] Outliers removed by the Cochran test.

[b] Outliers removed by single Grubbs' test.

Table 9. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the low PAH level in oyster

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC O2	30.7[a]	85.4	74.9	52.1	69.6	52.7	79.5	76.4	73.9
	SFC O4	74.6[a]	85.7	80.1	58.6	82.0	56.1	76.2	81.5	72.1
1-MN	SFC O2	68.1	61.7[a]	81.6	51.7	59.4	71.7	82.0	76.2	38.0
	SFC O4	64.2	299.8[a]	90.5	56.7	69.1	75.2	81.5	73.7	46.5
1-MP	SFC O2	45.1[a]	36.7[a]	21.8	12.1	19.9	18.0	25.0	24.6	12.9
	SFC O4	103.1[a]	22.5[a]	24.4	14.3	23.2	19.2	24.9	25.0	17.5
2,6-DMN	SFC O2	88.8[a]	29.9	30.5	61.9[a]	30.3	23.8	36.0	30.7	24.7
	SFC O4	231.7[a]	28.6	35.8	30.2[a]	36.9	26.1	35.6	36.4	21.3
3-MC	SFC O2	24.6	29.7	34.2	21.1	25.0	27.5	30.4	28.1	23.0
	SFC O4	27.4	27.0	34.6	24.6	29.2	28.0	30.6	29.4	25.2
Ant	SFC O2	4.0[a]	6.2	7.5	2.3	1.4	5.4	9.1	4.7	—[b]
	SFC O4	9.8[a]	6.6	8.8	1.3	1.5	5.5	8.9	5.1	10.4[b]
BaA	SFC O2	9.8	12.7	12.6	5.6	8.6	11.1	13.5	11.3	9.5
	SFC O4	10.9	11.7	13.4	8.1	9.5	11.6	13.4	12.0	10.8
BaP	SFC O2	1.5	4.3	2.7	1.4	1.5	1.2	4.3	2.6	2.3
	SFC O4	1.8	3.3	3.0	1.8	1.7	1.4	4.3	2.8	2.8
BbF	SFC O2	7.3	10.0	8.8	6.6	7.7	8.9	9.3	8.8	7.4
	SFC O4	8.0	9.3	9.5	7.6	9.2	9.8	9.3	9.2	7.9
BghiP	SFC O2	3.3	4.7	4.4	3.2	3.6	4.0	4.7	4.2	4.0
	SFC O4	3.8	4.5	4.7	3.6	4.2	4.2	4.7	4.3	3.9
BkF	SFC O2	5.4	8.0	8.2	5.1	6.1	7.2	7.4	6.8	5.3
	SFC O4	6.4	7.4	9.1	5.9	7.3	7.8	7.3	7.4	5.8
Chr	SFC O2	39.8	46.7	45.9	32.7	39.2	43.7	46.2	42.4	37.0
	SFC O4	44.9	44.9	48.5	38.2	46.6	44.8	46.0	45.5	40.3
DBahA	SFC O2	3.4	5.2	4.1	3.1	3.7	4.0	4.8	4.2	4.7
	SFC O4	3.8	4.3	4.3	3.6	4.4	4.1	4.7	4.4	3.8
Fln	SFC O2	11.4	13.1	14.6	8.1	10.6	10.3	14.1	12.8	12.2
	SFC O4	12.4	14.6	14.6	10.4	12.4	10.9	13.9	14.0	14.5
Flt	SFC O2	22.2	23.2	23.2	19.5	19.4	26.5	22.9	21.0	16.2
	SFC O4	24.5	22.3	25.1	13.5	23.0	28.2	23.5	22.6	19.8
IcdP	SFC O2	3.4	5.0	4.5	3.3	3.7	4.3	4.7	4.3	4.8
	SFC O4	4.0	4.5	5.0	3.7	4.4	5.4	4.6	4.4	4.2
Naph	SFC O2	64.1	67.4	79.8	61.6	59.3	71.1	79.2	74.2	74.4
	SFC O4	49.1	81.0	79.7	63.7	62.8	72.8	76.5	80.8	79.6
Phe	SFC O2	35.0	47.6	44.2	40.7	39.6	33.7	46.0	43.5	38.8
	SFC O4	37.9	47.7	49.1	36.8	47.2	36.7	45.4	46.4	32.6
Pyr	SFC O2	33.3	34.6	36.6	26.0	30.3	32.8	37.6	33.8	30.0
	SFC O4	34.8	36.6	39.0	30.1	36.5	34.3	37.1	35.8	33.3

[a] Outliers removed by the Cochran test.

[b] Removed due to the reported integration issues.

Table 10. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the mid PAH level in oyster

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC O1	96.8[a]	81.8	69.8	40.2	63.5	56.5	75.6	74.5	78.2
	SFC O7	31.3[a]	80.0	82.6	47.5	71.7	61.6	76.9	73.7	69.8
1-MN	SFC O1	84.8	86.4	109.9	35.5	75.9	96.1	107.4	102.2	45.4
	SFC O7	79.3	116.3	152.8	104.4	77.8	100.9	101.2	111.3	46.0
1-MP	SFC O1	500.3[a]	40.9	45.4	24.2	37.8	37.2	47.0	48.5	30.5
	SFC O7	91.8[a]	44.7	48.7	31.1	42.3	37.3	44.2	46.9	31.3
2,6-DMN	SFC O1	573.4[a]	71.6	54.5	14.6[a]	53.0	46.2	64.0	63.9	50.9
	SFC O7	179.1[a]	84.1	56.2	93.4[a]	58.4	49.1	58.3	54.9	45.2
3-MC	SFC O1	73.9	89.6	94.0	57.5	72.3	83.8	88.7	83.2	67.5
	SFC O7	75.8	79.1	98.6	74.5	74.8	82.3	83.9	86.6	69.8
Ant	SFC O1	21.9[a]	11.0	11.9	1.7	1.5	8.5	13.7	6.2	11.0[b]
	SFC O7	6.4[a]	9.4	13.0	1.8	0.5	7.2	12.6	6.6	24.8[b]
BaA	SFC O1	15.6	20.6	19.6	10.3	12.9	19.3	21.9	18.5	15.4
	SFC O7	15.3	19.6	21.1	12.8	11.4	18.4	20.3	19.0	16.9
BaP	SFC O1	3.4	7.0	5.7	2.7	2.8	3.1	9.0	4.7	5.6
	SFC O7	2.9	5.7	6.3	3.2	3.0	2.7	8.2	5.0	5.8
BbF	SFC O1	21.3	28.7	26.1	18.5	22.8	28.3	27.0	24.7	21.4
	SFC O7	23.2	26.1	27.4	23.9	23.6	25.9	25.0	25.9	22.1
BghiP	SFC O1	6.5	9.8	8.6	5.9	7.3	8.8	9.4	8.7	8.8
	SFC O7	6.7	8.2	9.4	7.7	7.5	8.5	9.0	9.0	7.5
BkF	SFC O1	13.9	19.8	19.6	12.2	15.1	19.0	18.1	17.2	14.0
	SFC O7	14.2	18.4	19.9	15.9	16.0	21.0	17.1	17.7	14.3
Chr	SFC O1	75.1	93.0	87.7	61.2	74.7	87.9	89.8	85.8	71.7
	SFC O7	75.4	84.6	93.1	78.3	79.2	86.4	83.8	87.1	73.5
DBahA	SFC O1	6.9	10.4	8.6	6.2	7.3	8.5	9.5	8.7	11.3[a]
	SFC O7	7.0	8.5	9.2	7.4	7.5	8.3	8.9	8.6	7.3[a]
Fln	SFC O1	18.1	25.1	22.5	14.6	16.8	18.0	23.2	21.5	19.7
	SFC O7	17.8	23.1	24.2	18.2	18.7	18.0	21.7	21.4	22.4
Flt	SFC O1	42.0	46.5	44.4	23.0	37.4	58.4	47.5	42.4	33.0
	SFC O7	40.6	43.1	47.2	40.0	40.5	47.2	42.8	45.4	34.0
IcdP	SFC O1	6.9	9.9	9.0	6.1	6.9	9.1	9.4	8.8	9.7
	SFC O7	6.9	8.8	9.5	7.8	7.7	8.2	8.7	8.9	7.8
Naph	SFC O1	89.8	118.9	110.0	77.4	87.6	112.1	117.3	115.8	113.6
	SFC O7	82.6	112.8	125.0	101.6	88.4	113.9	110.2	117.7	116.4
Phe	SFC O1	65.0	96.6	87.5	58.3	76.0	71.8	89.9	89.1	75.3
	SFC O7	68.1	87.2	93.6	77.9	84.0	72.0	83.8	88.3	81.7
Pyr	SFC O1	59.2	71.1	68.8	45.2[a]	55.2	64.9	69.3	64.6	56.2
	SFC O7	57.8	65.9	70.5	59.1[a]	59.4	62.8	64.6	63.6	57.9

[a] Outliers removed by the Cochran test.

[b] Removed due to the reported integration issues.

Table 11. Collaborative study results (in µg/kg) obtained by the participating laboratories in blind duplicates fortified at the high PAH level in oyster

		Laboratory No.								
PAH	Sample ID	1	2	3	4	5	6	7	8	9
1,7-DMP	SFC O5	136.0[a]	56.6	66.6	38.2	65.5	54.5	78.4	72.0	75.8
	SFC O6	16.4[a]	53.3	66.8	53.7	68.3	56.5	84.9	85.3	73.2
1-MN	SFC O5	296.9	289.7	289.0	214.7	182.5	222.5	275.3	309.5	125.4
	SFC O6	212.6	280.2	285.0	231.3	194.8	218.6	253.2	243.9	134.3
1-MP	SFC O5	4082.3[a]	121.1	173.3	108.1	148.6	138.2	184.2	185.9	103.7
	SFC O6	219.8[a]	130.0	184.0	150.7	160.5	130.8	203.2	217.5	135.1
2,6-DMN	SFC O5	2790.3[a]	190.4	154.6	339.2[a]	164.7	134.1	179.9	145.3	130.8
	SFC O6	331.7[a]	187.9	167.7	541.4[a]	168.9	127.6	173.9	159.6	173.0
3-MC	SFC O5	191.0	180.2	218.1	146.1	172.2	195.0	215.8	215.8	184.4
	SFC O6	214.7	199.9	233.3	165.6	185.1	194.0	226.0	217.2	187.6
Ant	SFC O5	59.3[a]	35.3	39.8	14.9[b]	9.1	26.8	52.1	30.0	12.0[b]
	SFC O6	136.2[a]	41.1	47.3	41.4[b]	10.3	29.0	54.7	32.1	44.1[b]
BaA	SFC O5	66.3	67.8	80.4	47.1	55.0	71.6	87.3	78.5	71.7
	SFC O6	74.8	79.2	82.3	50.0	60.7	71.9	91.1	80.8	72.4
BaP	SFC O5	16.9	25.5	32.0	14.7	14.8	12.7	42.4	25.8	28.4
	SFC O6	20.2	28.4	33.9	16.8	17.4	11.7	43.8	27.0	31.0
BbF	SFC O5	75.1	76.8	85.2	62.9	74.8	98.2	87.4	87.0	79.6
	SFC O6	78.2	86.3	87.9	70.4	78.8	101.7	91.6	88.7	79.5
BghiP	SFC O5	17.4	18.5	21.7	14.9	17.2	19.7	22.4	20.6	19.0
	SFC O6	19.0	21.2	22.2	16.5	18.6	19.8	23.5	20.9	19.8
BkF	SFC O5	55.5	59.2	65.7	46.9	54.2	71.3	67.3	66.5	60.5
	SFC O6	59.7	69.6	69.9	52.5	58.3	78.7	70.4	66.7	58.4
Chr	SFC O5	197.8	191.9	214.9	157.9	183.2	207.8	220.3	218.3	199.0
	SFC O6	214.2	210.7	226.5	176.6	194.5	207.2	231.4	219.4	201.5
DBahA	SFC O5	15.0	15.8	19.2	12.0	13.8	14.8	18.3	16.5	15.4
	SFC O6	16.2	17.4	18.4	13.4	15.0	15.5	19.2	16.8	15.3
Fln	SFC O5	51.0	63.5	61.1	36.9	48.3	48.8	66.4	62.0	70.8
	SFC O6	55.5	68.0	65.2	36.2	49.7	46.3	69.0	57.4	70.5
Flt	SFC O5	132.5	121.3	124.8	76.4	106.9	136.5	131.5	129.9	102.6
	SFC O6	127.3	135.3	130.6	92.8	113.9	126.6	137.8	131.5	115.1
IcdP	SFC O5	18.1	19.1	22.4	15.4	18.1	20.1	21.8	21.6	18.8
	SFC O6	19.5	21.4	22.3	17.1	19.2	22.0	23.1	21.6	19.2
Naph	SFC O5	214.6[a]	201.4	205.5	146.5	157.7	180.5	209.7	213.3	238.0
	SFC O6	171.1[a]	215.4	210.1	146.2	158.2	175.8	215.3	207.8	221.1
Phe	SFC O5	137.6[a]	208.6	203.1	192.7	183.8	168.7	222.5	224.5	196.8
	SFC O6	214.4[a]	227.0	217.7	189.6	194.1	158.2	232.7	222.1	220.4
Pyr	SFC O5	160.0	158.4	169.5	125.3	142.3	164.7	179.8	175.1	161.1
	SFC O6	173.2	179.1	179.6	140.1	151.2	154.6	187.4	176.0	163.4

[a] Outliers removed by the Cochran test.

[b] Removed due to the reported integration issues.

Table 12. Recoveries (%) of the studied PAHs in oyster obtained by a collaborator storing the test samples SFC O1–O7 at –20°C

PAH	SFC O1	SFC O2	SFC O3	SFC O4	SFC O5	SFC O6	SFC O7	Mean	RSD, %
1,7-DMP	93	95	93	102	90	107	92	96	6.3
1-MN	102	102		98	124	98	111	106	9.5
1-MP	97	99		100	93	109	94	99	5.8
2,6-DMN	85	77		91	65	71	73	77	13
3-MC	92	94		98	96	97	96	95	2.1
Ant	**41**	**47**		**51**	**50**	**53**	**44**	**48**	**9.6**
BaA	74	75		80	78	81	76	77	3.5
BaP	**47**	**51**		**56**	**52**	**54**	**50**	**52**	**6.3**
BbF	82	88		92	87	89	86	87	3.7
BghiP	87	83		87	82	84	90	86	3.4
BkF	86	85		92	89	89	88	88	2.9
Chr	86	85		91	87	88	87	87	2.5
DBahA	87	84		89	82	84	86	85	2.7
Fln	86	86		93	83	77	86	85	6.3
Flt	85	84		90	87	88	91	87	3.2
IcdP	88	85		88	86	86	89	87	1.6
Naph	93	93		101	95	92	94	95	3.4
Phe	89	87		93	90	89	88	89	2.2
Pyr	86	85		89	88	88	85	87	2.2

Table 13. Recoveries (%) of the studied PAHs in oyster obtained bya collaborator storing the test samples SFC O1–O7 at –70°C

PAH	SFC O1	SFC O2	SFC O3	SFC O4	SFC O5	SFC O6	SFC O7	Mean	RSD, %
1,7-DMP	94	99	108	95	98	106	96	100	5.4
1-MN	107	109		109	110	101	101	106	3.8
1-MP	94	100		100	92	102	88	96	5.5
2,6-DMN	85	90		89	80	77	78	83	6.8
3-MC	99	101		102	96	100	93	99	3.5
Ant	**92**	**91**		**89**	**87**	91	84	89	3.3
BaA	88	90		90	87	91	81	88	4.0
BaP	**90**	**87**		**86**	**85**	88	82	86	3.0
BbF	90	93		93	87	92	83	90	4.2
BghiP	94	95		94	89	94	90	93	2.7
BkF	91	92		91	90	94	85	90	3.1
Chr	90	92		92	88	93	84	90	3.8
DBahA	95	96		95	92	96	89	94	3.1
Fln	93	94		93	88	92	87	91	3.2
Flt	95	92		94	88	92	86	91	4.1
IcdP	94	93		93	87	92	87	91	3.5
Naph	94	99		96	93	96	88	94	3.8
Phe	90	92		91	89	93	84	90	3.6
Pyr	92	94		93	90	94	86	91	3.3

Candidates for 2016 Method of the Year

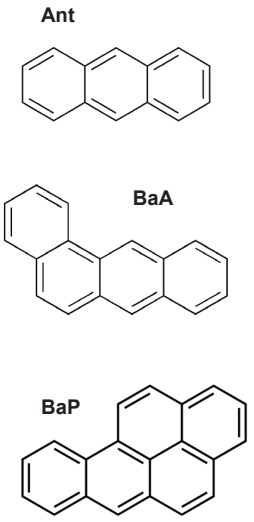

Figure 1. Structures of anthracene (Ant), benzo[*a*]anthracene (BaA), and benzo[*a*]pyrene (BaP).

blank PAH levels were too high to participate in the collaborative study and to conduct low-level PAH analysis in general. This was typically due to their location (high environmental contamination) and/or their laboratory contamination. Some participants were able to reduce the reagent (procedure) blank contamination by moving the sample preparation (extraction, cleanup, and evaporation) away from oil pumps, such as MS rough pumps. The method requires heating of the used salts and recommends heating of glassware. Solvents, plastic material, and equipment may also be sources of PAHs, including polypropylene centrifugation/extraction tubes. As one collaborator discovered, simple testing of polypropylene tubes using ethyl acetate wash/extraction may not reveal PAH contamination. However, the extraction dynamic during the actual procedure can release potentially present PAHs into the extract when the tubes get heated due to the exothermic reaction caused by addition of $MgSO_4$ to the water-containing extraction mixture. For this and other potential contamination reasons, it is highly important to analyze a reagent blank with every sample batch.

In addition to the contamination issues, another problem faced by laboratories less experienced in PAH analysis was optimization of the evaporation conditions to prevent losses of volatile analytes, especially naphthalene. Isooctane is used as a keeper in both evaporation steps, but it did not prevent significant losses of volatile PAHs in the second evaporation step in certain laboratories. For this reason, the study direction team recommended addition of 1–2 mL of ethyl acetate to the SPE eluent for a better control of the final evaporation process, which helped in most cases and was added as a recommendation to the method procedure.

Sixteen laboratories entered the qualification phase, but only 10 of them (listed in the *Acknowledgments* section) completed the qualification successfully and/or continued in the study. In many cases, the reason why a participant did not complete the qualification phase was the availability of resources and not the ability to qualify for the study.

Collaborators' Comments

Most of the study participants commented very positively on the speed and ease of use of the method, especially laboratories currently analyzing PAHs with much more labor-intensive and time-consuming methods.

The most frequently reported sources of PAH contamination were salts (which have to be muffled and stored appropriately). Some participants had problems with PAH sources in their laboratories caused by the use of oil pumps in the vicinity of the space used for the sample preparation. As noted above, one collaborator discovered PAHs in polypropylene centrifuge tubes used for practice sample analysis (*Note*: All tubes and containers used for the test sample storage and preparation were pretested by the study direction team).

Several collaborators initially had problems with optimization of the evaporation steps to prevent losses of volatile PAHs. As noted above, the addition of ethyl acetate in the second evaporation step resolved this issue in most cases. The majority of study participants used evaporation with a gentle stream of nitrogen at room temperature or a maximum of 40°C.

Collaborators noticed differences in the color of extracts of oyster blank versus fortified samples stored for several months in a freezer at –20°C. The blank sample produced a dark green extract, whereas the same blank sample fortified with PAHs gave a yellow-brown extract. This was not observed for oyster samples stored for a shorter period of time and/or stored at –70°C. As discussed below, this observation could be linked to degradation issues in oysters. Also, the participants noted that oyster extracts were generally dirtier than shrimp and mussel extracts, which affected chromatography in some cases.

One collaborator reported the use of a mechanical shaker instead of hand-shaking. Mechanical shaking is generally preferred by routine testing laboratories, and this method modification is acceptable as long as a vigorous and effective shaking (up and down in the tube) is ensured.

Collaborative Study Results

Tables 3–11 provide the collaborative study results obtained by the participating laboratories in three blind duplicates (low, mid, and high fortification level) in shrimp, mussel, and oyster. Results from Laboratory No. 10 are presented only for mussel because the Study Directors excluded their oyster and shrimp data sets due to calibration (standard preparation) issues.

1,7-DMP was used as a homogenization check and was added to blank mussel and oyster samples at 40 and 80 µg/kg, respectively, during the homogenization step. The mean concentration value obtained for 1,7-DMP by all participants (except for sample SFC M4 lost by Laboratory No. 6 and the result for sample SFC M3 from Laboratory No. 1, which was removed as an apparent outlier) in all seven test mussel samples (three blind duplicates and one blank) was 38.8 µg/kg (RSD = 21.5%, n = 68), which corresponds to mean recovery of 97.0%. In the case of oysters, the mean concentration value obtained for 1,7-DMP by all participants (except for Laboratories 1 and 10, for which all 1,7-DMP results were eliminated as outliers in the Grubbs' tests applied

to the blind duplicates) in all seven test samples was 70.5 µg/kg (RSD = 16.3%, n = 56), which corresponds to mean recovery of 88.1%. These results indicate that the mussel and oyster samples sent to the study participants had good homogeneity. 1,7-DMP was also added to all shrimp test samples at 20 µg/kg during the fortification step conducted by the study participants. The mean concentration value was 22.1 µg/kg (RSD = 19.0%, n = 63), corresponding to mean recovery of 111%. Statistical results for 1,7-DMP obtained in blind duplicate samples are summarized together with the other analytes in Tables **2014.08B–D**.

Tables **2014.08B–D** provide statistical results obtained for the studied analytes at three different concentration levels in shrimp, mussel, and oyster after elimination of statistical outliers (highlighted in Tables 3–11).

Eight to 10 valid results were obtained for the majority of determinations. Mean recoveries of all tested analytes at the total of five different concentration levels were all in the range of 70–120%: 83.8–115% in shrimp, 77.3–107% in mussel, and 71.6–94.6% in oyster, except for a slightly lower mean recovery of 68.6% for BaA fortified at 25 µg/kg in oyster (RSD_r: 5.84%, RSD_R: 21.1%) and lower mean recoveries for Ant and BaP in oyster at all three fortification levels (50.3–56.5% and 48.2–49.7%, respectively).

The lower mean recoveries of Ant and BaP were linked to degradation of these analytes in oyster samples stored at –20°C (*see* the discussion about degradation issues below), which also resulted in lower reproducibility (RSD_R values in the range of 44.5–64.7% for Ant and 40.6–43.5% for BaP). However, the repeatability was good (RSD_r of 8.78–9.96% for Ant and 6.43–11.9% for BaP), and the HorRat values were acceptable (1.56–1.94 for Ant and 1.10–1.45 for BaP).

In all other cases, repeatability, reproducibility and HorRat values were as follows:

(*1*) Shrimp: RSD_r 1.40–26.9%, RSD_R 5.41–29.4%, HorRat 0.22–1.34;

(*2*) Mussel: RSD_r 2.52–17.1%, RSD_R 4.19–32.5%, HorRat 0.17–1.13; and

(*3*) Oyster: RSD_r 3.12–22.7%, RSD_R 8.41–31.8%, HorRat 0.34–1.39.

Overall, the results of the collaborative study demonstrate that the method is fit-for-purpose to determine PAHs and their alkyl homologs in seafood samples.

Degradation Issues

The Study Directors reported problems with lower recoveries for Ant and BaP in oyster samples stored at –20°C to the SPSC PAH Working Group and the AOAC Methods Committee on PAHs when they discovered a significant difference between results for these two analytes obtained in two different participating laboratories storing the samples at two different freezer temperatures of –20 and –70°C (*see* recovery results in Tables 12 and 13, respectively). Due to the limited availability and cost of oyster samples, it was decided to continue with the study and not proceed with preparation of new study test samples that would be fortified by collaborators on the day of the analysis as was done for shrimp (*Note*: in the case of shrimp, overall lower recoveries were obtained for all studied PAHs depending on the shrimp sample storage conditions).

The laboratory storing samples at –20°C analyzed another set of oyster samples about 1.5 months after the first set and confirmed the lower recoveries for Ant and BaP. This collaborator also made another interesting observation related to the color difference between extracts obtained in the first set (all dark green) and the second set (the dark green extract was produced only for the blank sample), whereas all extracts of fortified samples were yellow-brown. This observation, which was later confirmed by additional study participants, indicates matrix changes caused by the presence of PAHs and accompanied by selective losses of Ant and BaP. In addition to these two analytes, BaA also showed lower recoveries (around 70%) in oysters when compared to the rest of the studied PAHs.

Furthermore, a closer examination of the results obtained for Ant, BaP, and BaA in mussel also show somewhat lower mean recoveries of these three analytes (around 80%) when compared to the other analytes. These mean results do not include data obtained for BaP in mussel test samples by Laboratory No. 6, which analyzed the test samples about a year later than most other participants. All Laboratory No. 6 BaP results in mussel test samples showed very good repeatability within the duplicates but were eliminated as Grubbs' test outliers because they were significantly lower than the results obtained by other laboratories. No other data obtained by Laboratory No. 6 were identified as outliers using the Cochran or Grubbs' tests.

Figure 1 provides structures of Ant, BaA, and BaP showing that these PAHs contain the same moiety in terms of the linear 3-ring (anthracene) structure. This structural commonality and similar degradation behavior indicate that they could be substrates for the same enzyme(s). The significant differences in recoveries obtained for samples stored at –20°C versus –70°C (Tables 12 and 13, respectively) represent more supporting evidence for an enzymatic degradation being the most probable cause for the lower recoveries observed for these analytes in oyster (and mussel) test samples. To prevent degradation of these analytes in seafood matrixes during long-term storage, the Study Directors recommend storing homogenized samples at –70°C or lower. Unfortunately, this was not a feasible requirement for this collaborative study because the majority of the collaborating laboratories did not have this storage capability.

Acknowledgments

The Study Directors wish to thank the SPSC PAH Working Group (chaired by Gina Ylitalo from National Oceanic and Atmospheric Administration), the AOAC Methods Committee on PAHs (chaired by Tom Phillips, Maryland Department of Agriculture, Annapolis, MD), and AOAC INTERNATIONAL staff for the discussions about the study design, analyte selection, and stability issues as well as for the overall support provided to the collaborative study. Special acknowledgment goes to Jack Cochran (Restek Corp., Bellefonte, PA) for useful discussions about the PAH GC/MS analysis.

John Schmitz and Jack Jabusch (Covance Laboratories, Madison, WI) and Lucie Drabova and Jana Pulkrabova (ICT, Prague, Czech Republic) are acknowledged for technical advice and support during the preparation and the entire course of the study.

The Study Directors thank the Nutritional Chemistry and Food Safety business unit at Covance Laboratories for supplying and preparing PAH standards and spiking solutions; obtaining

blank mussel samples; homogenizing, weighing, and fortifying all samples; and shipping samples to the study participants.

Cambridge Isotope Laboratories (Tewksbury, MA) is acknowledged for providing discounted ^{13}C-PAH standard mixtures, Shrimp Alliance for supplying blank shrimp samples, and Jo Marie Cook (Florida Dept. of Agriculture and Consumer Services, Tallahassee, FL) for securing blank oyster samples for the study.

The Study Directors are very grateful to all laboratories that participated in the study and especially to the following collaborators, listed alphabetically based on their institution name:

Rolando Perez, Jason Betz, and Steven Perez, Adpen Laboratories, Inc., Jacksonville, FL

Ken Keide, Mark Misunis, and Jian Wang, Canadian Food Safety Inspection Agency, Calgary Laboratory, AB, Canada

Jack Jabusch and John Schmitz, Covance Laboratories Inc., Madison, WI

Jason Stepp, Walter Hammack, and Jo Marie Cook, Florida Dept. of Agriculture and Consumer Services, Tallahassee, FL

Patricia Lopez-Sanchez, Radoslaw Lizak, and Thomas Wenzl, Institute for Reference Materials and Measurements, EU PAH Reference Laboratory, Geel, Belgium

Lucie Drabova and Jana Pulkrabova, Institute of Chemical Technology, Prague, Czech Republic

Joe Binkley, Doug Staples, and Scott Pugh, Leco Corporation, St. Joseph, MI

Bonita Taffe, Michigan Department of Community Health Bureau of Labs, Lansing, MI

Jan Rosmus, State Veterinary Institute, National PAH Reference Laboratory, Prague, Czech Republic

Katerina Bousova and Klaus Mittendorf, Thermo Fisher Scientific Food Safety Response Center, Dreieich, Germany

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(2) CONffIDENCE (2010) *Contaminants in Food and Feed: Inexpensive Detection for Control of Exposure*. http://www.conffidence.eu

(3) *Official Methods of Analysis* (2002) *Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis*, Appendix D. http://www.aoac.org/Official_Methods/Collaborative_Study_Validation_Guidelines.pdf

(4) AOAC Spreadsheet for Blind Duplicates 2.0. http://www.aoac.org/stats/

Expert Review Panel on Polycyclic Aromatic Hydrocarbons (PAHs)

OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT

ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final version of the Official Chair's Report for the Expert Review Panel on Polycyclic Aromatic Hydrocarbons (PAHs) held on Monday, September 8, 2014 during the AOAC Annual Meeting and Exposition at the Boca Raton Resort and Club, 501 East Camino Reel, Boca Raton, Florida 33432.

TOM PHILLIPS, MD DEPARTMENT OF AGRICULTURE

Expert Review Panel Chair

30 October 2014

Date

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EXPERT REVIEW PANEL MEETING ATTENDEES

Expert Review Panel Chair(s)
Tom Phillips, MD Department of Agriculture

Expert Review Panel Members
Mark Crosswhite, Florida Department of Agriculture
Julie Kowalski, RESTEK
Cheryl Lassitter, DOC, NOAA, NMFS, NSIL
Kai Liu, Eurofins
Jian Wang, Canadian Food Inspection Agency (CFIA)
Xiaoyan Wang, United Chemical Technologies, Inc. (UCT)
Stephen A. Wise, National Institute of Standards and Technology (NIST)

Not Present: Tracy Collier - (Alternate), NOAA (retired)
Not Present: Lowri de Jager, U.S. Food and Drug Administration

Method Authors
Kate Mastovska, Covance Laboratories
Jana Hajslova, Institute of Chemical Technology

AOAC Staff
Scott Coates
Deborah McKenzie
Tien Milor
La'Kia Phillips

Observers
Jana Huckrabolt, Institute of Chemical Technology
Nick Kosa, Bioo Scientific
Laszlo Torma,
Suramya Waidyanatha, NPT/NIEHS

EXPERT REVIEW PANEL, METHOD BACKGROUND, AND CONCLUSIONS

Criteria for Vetting Methods to be considered:
AOAC convened the *Official Methods of Analysis*^{SM} (OMA) Expert Review Panel for Polycyclic Aromatic Hydrocarbons (PAHs) on Monday, September 8, 2014 from 1:30pm to 4:00pm during the AOAC Annual Meeting and Exposition in Boca Raton, Florida. The purpose of the meeting was to 1) Review the Collaborative Study Manuscript/ OMAMAN-15: Determination Of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry (Study Director: Katerina Mastovska, Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA) and to 2) discuss First to Final Action requirements and feedback mechanisms. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, response to AOAC statistical review, summary shrimp corrected, DBAHA shrimp mid s9&s14 corrected, ICDP oyster mid o1&o7 corrected, and additional experiments with shrimp matrix.

Criteria for Vetting Experts and Selection Process:
The following nine (9) candidates and one (1) alternate were submitted for consideration by the Official Methods Board to evaluate candidate methods for Polycyclic Aromatic Hydrocarbons (PAHs) methods as per the Expert Review Panel (ERP) Policies and Procedures. The candidates were highly recommended by the Chemical Contaminants and Residues in Foods Community, have participated in various AOAC activities, including but limited to, Method Centric Committees that were formed under the legacy OMA pathway, and were vetted by the Official Methods Board. The experts are Tom Phillips, Cheryl Lassitter, Tracy Collier (Alternate Member), Kai Liu, Mark Crosswhite, Jian Wang, Lowri de Jager, Xiaoyan Wang, Julie Kowalski, and Stephen Wise. Tom Philips was vetted as the Expert Review Panel Chair.

ERP Orientation:
The ERP members have completed the mandatory AOAC Expert Review Panel Orientation Webinar on Wednesday, July 16, 2014.

Expert Review Panel Meeting Quorum
The meeting of the Expert Review Panel was held in person. A quorum is the presence of seven (7) members or 2/3 of the total vetted ERP, whichever is greater. Eight (8) out of the nine (9) voting members were present and therefore met a quorum to conduct the meeting.

Standard Method Performance Requirements (SMPRs): N/A

Conclusion:
The Expert Review Panel reviewed OMAMAN-15: Determination of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry and adopted this method for First Action Official Method status by a unanimous decision.

Subsequent ERP Activities:
ERP members will continue to evaluate the method for 2 years.

MEETING MINUTES

I. Welcome and Introductions

The Expert Review Panel Chair, Tom Phillips welcomed Expert Review Panel members and initiated introductions. The Chair discussed with the panel the goal of the meeting.

II. Review of AOAC Volunteer Policies

Deborah McKenzie presented an overview of AOAC Volunteer Policies, Volunteer Acceptance Agreement and and Expert Review Panel Policies and Procedures which included Volunteer Conflicts of Interest, Policy on the Use of the Association, Name, Initials, Identifying Insignia, Letterhead, and Business Cards, Antitrust Policy Statement and Guidelines, and the Volunteer Acceptance Form (VAF). All members of the ERP were required to submit and sign the Volunteer Acceptance Form.

III. Expert Review Panel Process Overview and Guidelines

Deborah McKenzie presented an overview of the Expert Review panel process. The presentation included information regarding method submission, recruitment of ERP members, composition and vetting expertise, method assignments, meeting logistics, consensus, First Action to Final Action requirements, method modifications, publications, and documentation.

IV. Review of Methods

All members of the ERP presented a review and discussed the proposed collaborative study manuscript for the Determination of Polycyclic Aromatic Hydrocarbons (PAHs) In Seafood Using Gas Chromatography-Mass Spectrometry. The method authors are Katerina Mastovska of Covance Laboratories Inc., Nutritional Chemistry and Food safety, 3301 Kinsman Boulevard, Madison, WI 53704, USA. A summary of comments was provided to the ERP members.[1]

MOTION:
Motion by Kowalski; Second by Crosswhite to adopt this method as a First Action Official Method.
Consensus demonstrated by: 7 in favor, 1 opposed, and 0 abstentions.
Motion failed.[2]

Negative Vote Discussion: One member of the expert review panel voted against the motion. Due to the reviewer's comments, he inquired about the method not using a certified reference material for PAHs in seafood. Standard Reference Material (SRM) 1974b Mussel Tissue is mentioned as part of the qualification of the labs as a practice sample, but no data was reported using SRM 1974b for validation of the proposed method. The availability of SRM 1974c (which has replaced SRM 1974b) provided an excellent opportunity to use a CRM to validate an AOAC method. The discussion of the Expert Review Panel concluded that the use of a certified reference material was not required and did not delineate scientific reasoning to not move the method forward. This method was created in an effort to address an emergency response to the gulf oil spill. The information provided in reference to the selection of the 19 target PAH compounds and the matrices selected were noted in the Fitness for Purpose statement established by the Stakeholder Panel on Petroleum Contaminants in Seafood in 2010. The ERP captured a revote.

[1] Attachment 1: Summary of Expert Reviewer Comments for OMAMAN-15

[2] *Method must be adopted by unanimous decision of ERP on first ballot, if not unanimous, negative votes must delineate scientific reasons. Negative voter(s) can be overridden by 2/3 of voting ERP members after due consideration.*

MOTION:
Motion by Kowalski; Second by Crosswhite to adopt this method as a First Action Official Method.
Consensus demonstrated by: 8 in favor, 0 opposed, and 0 abstentions (Unanimous).
Motion Passed.

V. **Discuss Final Action Requirements for First Action Official Methods** *(if applicable)*
No further action was discussed at this time.

VI. **Adjournment**

	Summary of Method
ER 1	PAHs in homogenized seafood's are extracted with EtOAc:water. Extracts are cleaned with SPE technique before GC-MS analysis.
ER 2	This method utilizes solvent extraction of a homogenized sample followed by a silica-SPE procedure. The eluant is introduced into a GC-MS or GC-MS/MS in either SIM or MRM modes. Issues encountered by participating laboratories are typical of PAH analyses including loss of more volatile PAHs during evaporation and background PAH contamination. This method achieved good sensitivity, accuracy and precision and has Limits of detection/quantification that are lower than the levels of concern in seafood samples set by regulatory agencies.
ER 3	The method describes analysis of 19 PAHs and alkylated PAHs via GC-MS. Sample preparation involves solvent extraction followed by salting out partitioning with silica SPE cleanup. Method performance criteria are set instead of prescribing specific products/instruments needed to successfully complete analysis. Criteria address analytes recovery, matrix cleanup, calibration quality, chromatographic separation and detector sensitivity.
ER 4	This method presented a procedure to determine 19 selected polycyclic aromatic hydrocarbons (PAHs) and their relevant alkyl homologous in seafood using fast sample preparation followed by GC-MS analysis. The sample preparation included two steps: (1) extraction using water-ethyl acetate and salt-out by anhydrous magnesium sulfate and sodium chloride; (2) clean-up by silica gel SPE. GC-MS was very common and practical instrumentation for PAHs analysis. The method is simple, fast, accurate, robust and is easy to follow.
ER 5	Collaborative study conducted to determine selected PAHs and relevant alkyl homologues in seafood matrices using a fast sample preparation method followed by analysis with gas chromatography-mass spectrometry (GC-MS).
ER 6	This is a GC-MS method with C13 labeled internal standards; sample preparation is a QuEChERS approach followed by SPE cleanup. The method was designed around performance-based criteria regarding the GC separation, SPE, and evaporation steps.
ER 7	PAHs in seafood samples (10 g, hydrated with 5 or 10 mL water) are extracted into 10 mL ethyl acetate with the aid of partitioning salts (4 g magnesium sulfate and 2 g sodium chloride). 5 mL of the ethyl acetate extract is concentrated down and cleaned with 1 g silica gel SPE cartridge, the eluate is concentrated and solvent exchanged to 0.5 mL isooctane, and analyzed by GC/MS.
ER 8	good

	Method Scope/Applicability
ER 1	Applicable to seafood's such as mussel, oyster, and shrimp.
ER 2	This method provides determination of PAH and PAH analogues in shrimp, mussels and finfish which are representative of this class of compounds. The method achieves limits of detection well below the regulatory levels of concern.
ER 3	Scope of the method includes 19 specific PAHs in seafood. The matrices tested, shrimp, oyster and mussel, are typically 5% lipid content and below (USDA Nutrient database). Lipid content of commodities amendable for this method is an important consideration and should be addressed in the text of the method. Higher fat samples are addressed briefly in the method, indicating that a reduction of volume of extract should be applied to the silica SPE cartridge. This is a reasonable modification to the method but has implications for overall detectability, especially for BaP. It is possible that to meet fat removal criteria, modifications for calibration curves and/or sample preparation will need to be made. Modifications may be significant and therefore some comment on an upper limit of the lipid content applicable for the method as written would be useful.
ER 4	The method covers 19 selected PAHs in shrimp, mussel, and oyster with analytical ranges of fit for purpose. The scope should be easily expanded to include more analytes and applied to a verity of seafood or processed ones.
ER 5	All Federal, State and Commercial laboratories analyzing PAHs in seafood.
ER 6	The methods is intended for seafood that would accumulate PAHs and has been tested using mussel, oyster, and shrimp matrices.
ER 7	The method is capable to detect 19 selected PAHs quantitatively in shrimp, oyster and mussel samples.
ER 8	good

	General Comments
ER 1	This study successfully addressed the urgent need for a reliable approach to analyzing various PAHs in potentially contaminated seafood's. Method procedures are easy to follow and not time-consuming.
ER 2	The validation procedure outlined in this document was comprehensive and the results demonstrated that the method was accurate and rugged. This method is a significant improvement over current regulatory methods. It is significantly faster, requires less organic solvent and produces less waste, less labor intensive and is easier to perform that the current method, while fulfilling required method performance benchmarks.
ER 3	none
ER 4	The collaborative study report was a very clear presentation. The experiment or study was thorough and well designed. Performance-based criteria led to a robust method for analysis of PAHs in seafood. The method was practical and fit for purpose. Results from collaborative study supported the method performance and demonstrated that the method was fit-for-purpose to determine PAHs and their alkyl homologues in seafood.
ER 5	Method is well-written but needs more specificity in select sections. For example, on page 6, under (5), it is stated all analytes of reagent blanks must be below the concentrations in the lowest calibration standard. Needs more clarification....how far below? Also, since stability of some PAHs was questionable, a Stability Study needs to be carried out with PAH standards stored at varying temperatures and times. The Safety Section must be in the front of the method since safety is more important than any other part of the protocol.
ER 6	None
ER 7	The method is quick, easy to use, and allows flexibility in method development. It demonstrated good GC separations of isomer pairs, excellent recoveries and reproducibility were achieved except for 2 compounds in oyster which might degrade at -20 C, no degradation was observed when oyster was stored at -70 C, however it's not very practical for many labs to maintain such low storing temperature.
ER 8	Very good method

	Method Clarity
ER 1	Good clarity throughout the manuscript.
ER 2	The method is well written and easily understood. The instructions are clear and I found no ambiguities.
ER 3	The method as written is clean with only a few instances for improvement. Performance criteria and how to evaluate the criteria are nicely described.
ER 4	The method procedure is well described and steps are easy to follow.
ER 5	Method is generally clear but needs more specificity in select areas.
ER 6	The method is clear and all the necessary information provided to reproduce and use the method. The authors should use correct nomenclature for the PAHs, i.e., Benzo[ghi]perylene not benzo[g,h,i]perylene
ER 7	The method is clearly described in the manuscript.
ER 8	good

	Pros/Strengths
ER 1	The organizer took great effort setting up the procedures to allow for individual lab's choice of various instruments, columns, SPE vendors, and evaporation techniques, as long as the lab passed the performance requirements. The procedures are straight forward and not hard to follow.
ER 2	Fairly easy method that requires significantly less sample preparation compared to other methods. Utilizes equipment and instrumentation that is widely available. Uses less flammable and toxic solvents than other methods. Sample handling is minimized which decreases the probability of environmental contamination.
ER 3	Strengths include: 1. easy to follow criteria for sample preparation evaluation 2. easy to follow instructions for solution and calibration solution preparation 3. some allowance for environmental background of the naphthalene 4. importance of monitoring blank is clearly stated
ER 4	The method is simple, fast, accurate, robust and is easy to follow.
ER 5	Well written, encompasses analyses of PAH compounds deleterious to humans at low levels, the calculations outlines on pages 14-15 are well written.
ER 6	A major strength is that the method is an isotope dilution (ID) GC-MS methods using C13 labeled internal standards for 13 of the 19 target PAHs. The sample preparation appears to be simplified compared to normal solvent extraction methods (Soxhlet, ACE, MAE). Another strength is the performance criteria required for the choice of GC column and the requirements to separate critical PAH isomers such as the benzofluoranthenes.
ER 7	The method is simple, fast, and easy to use. High sample throughput with little lab ware needed. Applicable to a variety of seafood matrices. Overall method performance are acceptable.
ER 8	no comment

	Cons/Weaknesses
ER 1	Isotope-Labeled mixed standards may be expensive or could be unavailable occasionally. Precision of results (all three levels) may have some room for improvement.
ER 2	Still requires some sample clean up, including a dry down step which if performed incorrectly could cause artificially low calculated concentrations for low molecular weight PAHs. Does not incorporate many alkyl homolog PAH compounds. These are often present at higher concentration in oil contamination and have similar toxicity to the PAHs. Addition of these compounds to the GC-MS method would increase the applicability and impact of the method. This could perhaps be done in the future
ER 3	Weaknesses include: 1. Method scope of 1 ug/kg LOQ of BaP was not tested as a fortification level. As I read the method, the lowest fortification level for BaP was 2 ug/kg. 2. Polypropylene tubes used for extraction will likely cause users of the method issues with PAH contamination. Discussion of alternatives would be helpful. 3. PAH GC-MS analysis has significant differences than typical analysis of most other types of compounds. Guidance for GC-MS parameters would likely be helpful for users of the method. These include parameters like inlet temperature, transfer line temperature, ion source temperature, column loadability and efficient flow conditions. 4. There is no recommendation on how to report data on chrysene and triphenylene if the recommended, but not required, 50% valley separation is not met. Can chrysene and triphenylene be reported together? 5. Ion ratios are mentioned as a requirement for identification but there is no indication as to the RSD value that is acceptable or some other qualification. 6. When a linear calibration curve is not possible, allowance for a "well-characterized" quadratic formula is made but with no discussion of what "well-characterized" means. Some guidance would be useful because some user will not be accustomed using quadratic calibration curves.
ER 4	A commercially available mix of standards suitable for the method is beneficial.
ER 5	The Safety Section must be in the front of the method since safety is more important than any other part of the protocol. Method must be more specific. Under Degradation Issues on page 18, the discussion emphasizes the need for a Stability Study. 18.2 megaohm water should be used for any GC/MS method (page 9, Section C). Need a statement that documented calibrations/reference checks were performed on all analytical equipment and instrumentation used in the collaborative study.
ER 6	A major weakness is that there is no validation using a certified reference materials for PAHs in seafood. Standard Reference Material (SRM) 1974b Mussel Tissue is mentioned as part of the qualification of the labs (p. 6) as a practice sample, but no data are reported using SRM 1974b for validation of the proposed method. The availability of SRM 1974c (which has replaced SRM 1974b) provided an excellent opportunity to use a CRM to validate an AOAC method. The use of only fortified/spiked samples for the method validation is a weakness. Spiked samples are sometimes the only option but in this

	case with SRM 1974 available, it could have been handled differently. The selection of the 19 target PAHs to determine could be questioned. The authors comment on criteria for separation of chrysene and triphenylene and also BaP and BeP, which is good, but perhaps triphenylene and BeP should have been included. There were no performance criteria for the separation of the alkyl-PAHs from potential isomers, e.g., with 3-MeChr targeted, how do you know if you are separating it from other methylchrysene or methyl-BaA isomers? The inclusion of several alkyl-PAHs is good, but will this really provide a method to look at the alkyl-PAHs, which in petroleum contaminated samples may be more abundant than the parent PAHs. Should there be a provision for looking at the alkyl-PAHs as a group by MS? Perhaps this is beyond the intended scope of this method.
ER 7	Fish, one of the most common seafood, was not covered in this method. Oyster needs to be stored at -70 C, which is not very easy for many labs to maintain.
ER 8	no comment

	Supporting Data Comments
ER 1	Great summary of results and provided complete statistical information. Would like to see more on linearity results.
ER 2	The supporting data indicates that the method is rugged and accurate.
ER 3	Nicely organized.
ER 4	na
ER 5	More specificity needed in quantitative parameters.
ER 6	In general, this is a good method, but needs further validation.
ER 7	Tested real samples. Additional experiments with shrimp matrix performed.
ER 8	good

	Method Optimization
ER 1	N/A
ER 2	Was well described and performed appropriately.
ER 3	Good.
ER 4	na
ER 5	The Safety Section must be in the front of the method since safety is more important than any other part of the protocol. Method must be more specific when outlining quantitative parameters. Under Degradation Issues on page 18, the discussion emphasizes the need for a Stability Study. 18.2 megaohm water should be used for any GC/MS method (page 9, Section C). Need a statement that documented calibrations/reference checks were performed on all analytical equipment and instrumentation used in the collaborative study.
ER 6	The method optimization is well done in particular the SPE cleanup. Could the use of an aminopropyl SPE be less sensitive than silica regarding deactivation by moisture content?
ER 7	Elution solvent volume for silica gel SPE cleanup is optimized. Increased water amount (10 mL) is used in shrimp samples to help shake and extract PAHs from more viscous shrimp samples.
ER 8	good

	Analytical Range
ER 1	Varies depending on the analyte. Generally, low is from 2 ppb to 25 ppb. High is 20 ppb to 250 ppb.
ER 2	Range varies and is dependent on the specific analyte. This is appropriate as lower molecular weight PAHs are generally found in higher concentrations than higher molecular weight PAHs. It is acceptable for this application as levels of concern for low molecular weight PAHs are significantly higher.
ER 3	Sufficient.
ER 4	BaP: 0.5 - 100 µg/kg (0.5, 1, 2, 5, 10, 20, 50, 100) Other PAHs: 1.25 - 250 µg/kg (1.25, 2.5, 5, 12.5, 25, 50, 125, 250) naphthalene: 2.5 - 500 µg/kg (2.5, 5, 10, 25, 50, 100, 250, 500)
ER 5	Good
ER 6	Suitable
ER 7	0.5 to 100 ug/kg for BaP and other lower-level PAHs; 1.25 to 250 ug/kg for higher-level PAHs; and 2.5 to 500 ug/g for naphthalene.
ER 8	good

	LOQ
ER 1	Not discussed in the method. Likely in the low ppbs.
ER 2	LOQ varies and is dependent on the specific analyte. This is appropriate as lower molecular weight PAHs are generally found in higher concentrations than higher molecular weight PAHs. It is acceptable for this application as levels of concern for low molecular weight PAHs are significantly higher.
ER 3	Please see comment above about spike levels and LOQ of BaP.
ER 4	Bap: 0.5 µg/kg Other PAHs: 1.25 µg/kg naphthalene: 2.5 µg/kg
ER 5	Good
ER 6	Suitable
ER 7	1 ug/kg for BaP and other lower-level PAHs; 2.5 ug/g for higher-level PAHs; and 5 ug/g for naphtalene.
ER 8	good

	Accuracy/Recovery
ER 1	Varies depending on the analyte. 70-120% mostly.
ER 2	Accuracy/Recovery is high and meets validation criteria.
ER 3	Good
ER 4	In shrimp: 83.8-115% In mussel: 77.3-107% In oyster: 71.6-94.6%, except for a lower mean recovery of 68.6% for benzo[α]anthracene (BaA) in oyster, and 50.3-56.5% and 48.2-49.7% for anthracene and beno[α]pyrene, respectively.
ER 5	Good
ER 6	As noted by the authors, the recoveries for the oyster tissue are low and probably inadequate.
ER 7	After excluding the outliers, the mean recoveries (8-10 labs) are in the range of 70-120% with a few exceptions which may due to the compound degradation in oyster samples stored at -20 C.
ER 8	good

	Precision
ER 1	Varies. Mostly around or below 10%, with one exception of 27% for low level 1-MN.
ER 2	Precision and reproducibility varies and is dependent on the specific analyte. The reported values meet the validation criteria
ER 3	Good
ER 4	In Shrimp: 1.40-26.9% In mussel: 2.52-17.1% In oyster: 3.12-22.7%
ER 5	Good
ER 6	I am not familiar with the expectations for precision for an AOAC method; however, the precision here appears to be adequate.
ER 7	Precision was excellent.
ER 8	good

	Reproducibility
ER 1	Varies. Mostly between 10%-20%.
ER 2	Precision and reproducibility varies and is dependent on the specific analyte. The reported values meet the validation criteria
ER 3	Good
ER 4	In Shrimp: 5.41-29.4% In mussel: 4.19-32.5% In oyster: 8.41-31.8%
ER 5	Good
ER 6	I am not familiar with the expectations for reproducibility for AOAC method; however, the reproducibility for this study appears to be inadequate for many of the more volativle PAHs (e.g., naphthalene) and particularly in the oyster tissue.
ER 7	Reproducibility was good except for a few compounds in oyster stored at -20 C.
ER 8	good

	System Suitability
ER 1	Not discussed.
ER 2	System is suitable
ER 3	Good
ER 4	na
ER 5	Were IDLs, MDLs and PQLs carried out on all instrumentation used in the Collaborative Study? If not, this should be performed and documented in the Method. Are there records of Intra-day, Inter-day variability? Are there records of Analyst variability? If so, the Method should state.
ER 6	No comments
ER 7	System check samples were analyzed.
ER 8	very good

	First Action Recommendation
ER 1	Yes.
ER 2	Yes
ER 3	Yes
ER 4	I recommend that the method, which has been gone through AOAC collaborative study successfully, for determination of PAHs in seafood using GC-MS be adopted Official Fist Action
ER 5	No
ER 6	Not yet....I think it needs validation with a natural matrix CRM such as SRM 1974c.
ER 7	Yes, with minor modifications (please see After First Action Recommendation)
ER 8	yes

	After First Action Recommendation
ER 1	Explore for ways to improve inter-lab precision RSD(R)%
ER 2	NO
ER 3	See comments above.
ER 4	na
ER 5	It may be helpful to refer to the FDA's LIB # 4475 to get a better feel for how the Method should be formatted and important quantitative data to include. The only exception here is the Safety Section is not in the front of this FDA LIB.
ER 6	As mentioned above, information on the method performance using SRM 1974c
ER 7	Fish samples should be analyzed in the future to see if this method is applicable to fish as well, especially those with high fat content. Was matrix effect significant? or the internal standards (13C PAHs) added to samples before extraction corrected the matrix effect of their corresponding PAHs? How about the alkyl PAHs that did not include their isotope labeled standards in this study? Should matrix matched calibration be more appropriate? I would suggest the study group to compare the PAH recoveries using this method and one of the other currently accepted methods to test an oyster reference material stored at - 20 C to show if the degradation of Ant and BaP is method dependent.
ER 8	no

AOAC Official Method 2014.09
Determination and Confirmation of Residues of 653 Multiclass Pesticides and Chemical Pollutants in Tea
GC-MS, GC-MS/MS, and LC-MS/MS
First Action 2014

Forty representative pesticides were selected based on guidance from the AOAC Method-Centric Committee on Pesticide Residues to conduct a multilaboratory validation study of the single-laboratory validated (SLV) method, using two representative brands of tea. The representative pesticides selected for the multilaboratory validation study of the SLV method are as follows: 2,4'-DDE, 4,4'-DDE, benalaxyl, bifenthrin, bromophos-ethyl, bromopropylate, chlorfenapyr, diflufenican, dimethenamid, fenchlorphos, picoxystrobin, pirimicarb, pirimiphos-methyl, procymidone, propyzamide, pyrimethanil, quinoxyfen, tefluthrin, tolclofos-methyl, and trifluralin by GC-MS and GC-MS/MS; acetochlor, benalaxyl, bensulide, butralin, chlorpyrifos, clomazone, diazinon, ethoprophos, flutolanil, imidacloprid, indoxacarb, kresoxim-methyl, monolinuron, picoxystrobin, pirimiphos-methyl, propoxur, quinoxyfen, tebufenpyrad, triadimefon, and trifloxystrobin by LC-MS/MS.

The LOQs for the 653 pesticides included in the SLV ranged from 0.03 to 1210 µg/kg. The LODs of the GC-MS method ranged from 1.0 to 500 µg/kg, and the corresponding LOQs ranged from 2.0 to 1000 µg/kg. The LODs of the GC-MS/MS method ranged from 1.0 to 900 µg/kg, and the corresponding LOQs ranged from 2.0 to 1800 µg/kg. The LODs of the LC-MS/MS method ranged from 0.03 to 4820 µg/kg, and the corresponding LOQs ranged from 0.06 to 9640 µg/kg.

Four-hundred eighty-two (482) of the 653 pesticides can be analyzed by GC-MS and GC-MS/MS, while 417 of the 653 pesticides can be analyzed by LC-MS/MS with LODs ≤100 µg/kg. There are 264 out of the 653 pesticides that can be analyzed by GC-MS, and 325 out of 653 by LC-MS/MS with LODs ≤10 µg/kg.

There are 270 pesticides that can be analyzed by both GC-MS and LC-MS/MS. Of these, there are 264 pesticides that can be analyzed by GC-MS and 247 by LC-MS/MS, with LODs ≤100 µg/kg for the GC-MS method. There are, however, 133 pesticides that can be analyzed by GC-MS and 200 by LC-MS/MS, with LODs ≤10 µg/kg.

A. Principle

Test samples are extracted with acetonitrile using a homogenizer, and the extracts are cleaned up with Cleanert TPT cartridge, EnviCarb/PSA or ECPSACB506 (UCT), or BE Carbon 500 g/PSA (Agilent) or InerSep GC/PSA (GL Sciences), or equivalent cartridges. The pesticides are eluted with acetonitrile-toluene (3 + 1, v/v), concentrated, dried, dissolved in the recommended solution, and analyzed by GC-MS, GC-MS/MS, or LC-MS/MS and quantified with a matrix-matched standard calibration curve.

B. Reagents

(**a**) *Solvents*.—Acetonitrile, toluene, and *n*-hexane (HPLC grade).

(**b**) *Acetonitrile-toluene.*—3 + 1 (v/v).

(**c**) *Ultrapure water*.--Obtained in a Milli-RO plus system together with a Milli-Q system (Millipore, (Bedford, MA, USA).

(**d**) *Anhydrous sodium sulfate*.—Analytically pure. Baked at 650°C for 4 h and stored in a desiccator.

C. Materials

(**a**) *SPE cartridges*.—Cleanert TPT (2000 mg, 12 mL; Agela, Tianjin, People's Republic of China) or EnviCarb/PSA (500 mg/500 mg, 6 mL; Supelco, Bellefonte, PA, USA) or equivalent.

(**b**) *SPE tube adapter*.—For 12 mL SPE tubes (57267), for 6 mL SPE tubes (57020-U; Sigma-Aldrich Shanghai Trading Co., Ltd, Shanghai, People's Republic of China) or equivalent.

(**c**) *Disposable flow control valve liners*.--For Visiprep TM-DL (57059; Sigma-Aldrich Shanghai Trading Co., Ltd).

(**d**) *Pear-shaped flask*.—80 mL (Z680346-1EA; Sigma-Aldrich Shanghai Trading Co., Ltd), or equivalent. *See* Figure **2014.09A**.

(**e**) *Reservoir*.—30 mL (A82030; Agela), or equivalent. *See* Figure **2014.09A**.

(**f**) *Centrifuge tube*.—80 mL.

(**g**) *Millipore filter membrane (nylon)*.—13 mm × 0.2 μm.

D. Preparation of Standard Solutions

(**a**) *Preparation of stock solutions.*--Accurately weigh 5-10 mg of individual pesticide and chemical pollutants standards (accurate to 0.1 mg) into a 10 mL volumetric flask. Dissolve and dilute to volume with methanol, toluene, acetone, acetonitrile, isooctane, etc., depending on each individual compound's solubility. All standard stock solutions are stored in the dark at 0-4°C and can be used for 1 year.

(**b**) *Preparation of mixed standard solution.*--Depending on properties and retention times of compounds, all compounds are divided into a series of groups. The concentration of each compound is determined by its sensitivity on the instrument for analysis. Mixed standard solutions are stored in the dark below 4°C.

(**c**) *Working standard mixed solution in matrix.*--Working standard mixture solution in matrix of pesticide and chemical pollutants is prepared by diluting an appropriate amount of mixed standard solution with blank extract, which has been taken through the method with the rest of the samples. Mix thoroughly. Used for plotting the standard curve.

Working standard mixture solution in matrix must be prepared freshly.

E. Apparatus and Conditions

(**a**) *GC-MS analysis.*—(*1*) *GC-MS system*.—Model 7890A gas chromatograph connected to a Model 5975C mass selective detector with electron ionization (EI) source and equipped with a Model 7683 autosampler and Chemstation data processing software system (Agilent Technologies, Wilmington, DE, USA), or equivalent.

(*2*) *Column*.--DB-1701 capillary column (30 m × 0.25 mm × 0.25 µm), or equivalent.

(*3*) *Column temperature*.—40°C hold 1 min, at 30°C/min to 130°C, at 5°C/min to 250°C, at 10°C/min to 300°C, hold 5 min.

(*4*) *Carrier gas*.--Helium, purity ≥99.999%, flow rate 1.2 mL/min.

(*5*) *Injection port temperature*.--290°C.

(*6*) *Injection volume*.--1 µL.

(*7*) *Injection mode*.--Splitless, purge on after 1.5 min.

(*8*) *Ionization mode*.—EI.

(*9*) *Ion source polarity*.--Positive ion.

(*10*) *Ionization voltage*.--70 eV.

(*11*) *Ion source temperature*.--230°C.

(*12*) *GC-MS interface temperature*.--280°C.

(*13*) *Solvent delay*.--14 min.

(*14*) *Ion monitoring mode*.--Selected ion monitoring (SIM), one quantifying ion and two qualifying ions are selected for each compound. The retention times, quantifying ions, qualifying ions, and the expected ion abundances for each of the 20 pesticides included in the study and heptachlor epoxide are listed in Table **2014.09A**. SIM acquisition parameters for ions monitored by GC-MS are shown in Table **2014.09B**.

(**b**) *GC-MS/MS analysis.*--(*1*) *GC-MS/MS system*.—Model 7890A gas chromatograph connected to a Model 7000B triple quadrupole mass spectrometer with EI source and equipped with a Model 7693 autosampler and Mass Hunter data processing software system (Agilent Technologies), or equivalent.

(*2*) Operating conditions are the same as for GC-MS with the exception that the ion monitoring mode is by selected reaction monitoring (SRM) and monitoring one precursor ion and two product ion transitions.

(*3*) The monitored ion transitions and the collision energies for the 20 pesticides of interest in the study and heptachlor epoxide are shown in Table **2014.09C**. The SRM acquisition partameters for the precursor

and product ion transitions monitored by GC-MS/MS are shown in Table **2014.09D**.

(**c**) *LC-MS/MS analysis.*—(*1*) *LC-MS/MS system*.—An Agilent Series 1200 HPLC system directly coupled to a 6430 triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source and Model G1367D autosampler with a Mass Hunter data processing software system (Agilent Technologies, Santa Clara, CA, USA). LC separation was achieved on a Zorbax SB-C18 column, 2.1 × 100 mm, 3.5 µm (Agilent Technologies) or equivalent.

(*2*) *Column*.--Zorbax SB-C18, 2.1 × 100 mm, 3.5 µm, or equivalent.

(*3*) *Mobile phase program and flow rate*.--*See* Table **2014.09E**.

(*4*) *Column temperature*.--40°C.

(*5*) *Injection volume*.--10 µL.

(*6*) *Ionization mode*.—ESI.

(*7*) *Ion source polarity*.--Positive ion.

(*8*) *Nebulizer gas*.--Nitrogen gas.

(*9*) *Nebulizer gas pressure*.--0.28 Mpa.

(*10*) *Ion spray voltage*.--4000 V.

(*11*) *Dry gas temperature*.--350°C.

(*12*) *Dry gas flow rate*.--10 L/min.

(*13*) Monitored ion transitions, collision energies, and fragmentation energies for the 20 pesticides and chlorpyrifos methyl are shown in Table **2014.09F**; and SRM acquisition parameters by LC-MS/MS for the precursor and product ion transitions monitored are shown in Table **2014.09G**.

(**d**) *Homogenizer*.—Rotational speed higher than 13500 r/min (report also in *g*-force units; T-25B, Janke & Kunkel, Staufen, Germany or equivalent).

(**e**) *Rotary evaporator*.—Buchi EL131 (Flawil, Switzerland) or equivalent.

(**f**) *Centrifuge*.—Centrifugal force higher than 2879 × *g* (Z320; B. HermLe AG, Gosheim, Germany or equivalent).

(**g**) *Nitrogen evaporator*.—EVAP 112 (Organomation Associates, Inc., New Berlin, MA, USA) or equivalent.

(**h**) *TurboVap*.—LV Evaporation System (Caliper Life Sciences, Hopkinton, MA,USA) or equivalent.

(**i**) *Visiprep 5-port flask vacuum manifold*.—RS-SUPELCO 57101-U (Sigma-Aldrich Shanghai Trading Co., Ltd). *See* Figure **2014.09B** or equivalent.

(**j**) *Variable volume pipet*.--10 µL, 200 µL, and 1 mL.

(**k**) *Balance*.--Capable of accurately measuring weights from 0.05 to 100 g within ±0.01 g.

F. Extraction and Cleanup Procedure

(**a**) *Sample extraction*.—(*1*) Weigh 5 g dry tea powder (accurate to 0.01 g) into an 80 mL centrifuge tube.

(*2*) Add 15 mL acetonitrile.

(*3*) Homogenize at 13500 r/min for 1 min.

(*4*) Centrifuge at 2879 × *g* for 5 min at room temperature.

(*5*) Transfer the supernatant into a pear-shaped flask.

(*6*) Reextract the sample with 15 mL acetonitrile, homogenize, centrifuge, and combine the supernatants from the two extractions.

(*7*) Concentrate the extract to approximately 1 mL in a rotary evaporator (or TurboVap) in a 40°C water bath.

(*8*) Place a pear-shaped flask in the vacuum manifold.

(*9*) Mount a Cleanert TPT cartridge onto the manifold.

(*10*) Add anhydrous sodium sulfate (approximately 2 cm) onto the Cleanert TPT packing material.

(*11*) Add 10 mL acetonitrile-toluene (3 + 1, v/v) to activate the cartridge.

(*12*) Stop the flow-through the cartridge when the liquid level in the cartridge barrel has just reached the top of the sodium sulphate packing.

(*13*) Discard the waste solution collected in the pear-shaped flask and replace with a clean pear-shaped flask.

(**b**) *SPE cleanup*.--(*1*) Load the concentrated extract from **F**(**a**)(*7*) into the conditioned Cleanert TPT cartridge collecting the eluate into the clean pear-shaped flask.

(*2*) Rinse the pear-shaped flask that contained the concentrated extract with 3 × 2 mL acetonitrile-toluene (3 + 1, v/v).

(*3*) Load the rinse into the cartridge when the level of the loading solution in the cartridge reaches the top of the anhydrous sodium sulfate packing.

(*4*) Connect a 30 mL reservoir onto the upper part of the cartridge using an adapter (*see* Figure **2014.09A**).

(*5*) Elute the cartridge with 25 mL acetonitrile-toluene (3 + 1, v/v).

(*6*) Evaporate the eluate to approximately 0.5 mL using a rotary evaporator (or TurboVap) in a 40°C water bath.

For GC-MS and/or GC-MS/MS analysis only:

(*7*) Add 40 µL heptachlor epoxide (internal stsandard; ISTD) working standard solution to the sample in **F**(**b**)(*6*).

(*8*) Evaporate to dryness under a stream of nitrogen in a 35°C water bath (or Turbo Vap).

(*9*) Dissolve the dried residue in 1.5 mL hexane, ultrasonicate the samples to mix, and filter through a 0.2 µm membrane filter. The sample is ready for GC-MS or GC-MS/MS analysis.

For LC-MS/MS analysis only:

(*10*) Add 40 µL chlorpyrifos methyl (ISTD) working standard solution to the sample prepared in **F**(**b**)(*6*).

(*11*) Evaporate to dryness under a stream of nitrogen in a 35°C water bath (or Turbo Vap).

(*12*) Dissolve the dried residue in 1.5 mL acetonitrile-water (3 + 2, v/v), ultrasonicate the samples to mix, and filter through a 0.2 µm membrane filter. The sample is ready for LC-MS/MS analysis.

G. Qualitative and Quantitative Analysis

(**a**) *Criteria for qualitative identification and confirmation.*—(*1*) Measure the retention time of the monitored peaks and match them with the same peaks on the pesticide standard chromatograms.

(*2*) Measure the ion abundances for the qualifier ions for the detected pesticides and verify that they are within the expected limits; if the peaks match, the presence of the pesticide is confirmed. *See* Table **2014.09H**.

(**b**) *Quantitative calculations.*—(*1*) Use instrument data processing software for GC-MS (SIM), GC-MS/MS, and/or LC-MS/MS to calculate a response ratio (measured adundance of pesticide/measured abundance of heptachlor epoxide for GC and chlorpyrifos methyl for LC) and construct a 5-point matrix-matched calibration curve of response ratio versus concentration of pesticide in standard solution.

(*2*) Using the regression data from the appropriate matrix-matched calibration curve, calculate the concentration of each pesticide found in the samples.

(*3*) If a validated computer system is not being used for calculations, follow the steps below:

(*a*) Measure the peak area of each respective standard level for each pesticide and the peak area of corresponding internal standard.

(*b*) Calculate the ratio of the analyte response to that of the internal standard.

(*c*) Run a linear regression analysis using the ratio of each pesticide at five different levels with no weighting or 1/x weighting, where x = concentration.

(*d*) Measure the peak area of each pesticide found in the sample and the peak area of corresponding internal standard.

(*e*) Calculate the amount of each pesticide in the solution injected from the standard curve.

(*f*) Calculate the amount of each pesticide present in the sample.

Test results should be retained to two decimal places or four significant digits.

Reference: *J. AOAC Int*. (future issue)

Posted: January 20, 2015

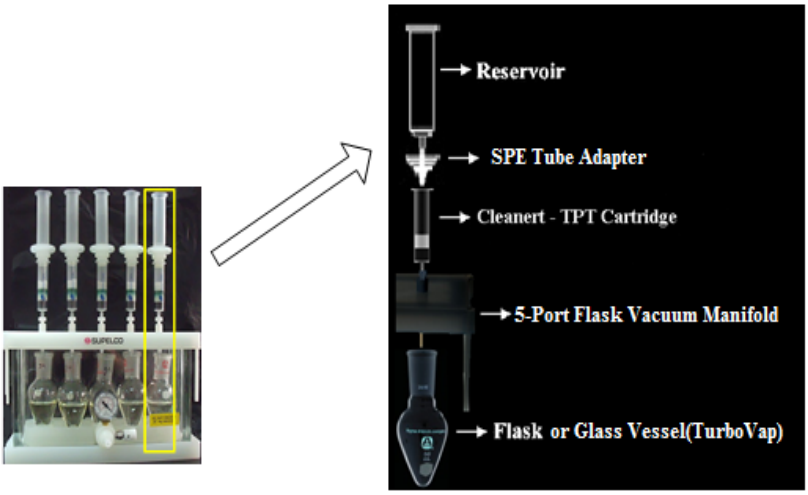


Figure 2014.09A. Solid-phase extraction equipment.

Table 2014.09A. GC-MS retention times, quantifying ions, qualifying ions, ion abundances, LODs, and LOQs for the 20 pesticides

No.	Pesticide	Retention time, min	Quantifying ion	Qualifying ion 1	Qualifying ion 2	LOQ, µg/kg	LOD, µg/kg
ISTD	Heptachlor-epoxide	22.15	353(100)	355(79)	351(52)		
1	Trifluralin	15.43	306(100)	264(72)	335(7)	20.0	10.0
2	Tefluthrin	17.35	177(100)	197(26)	161(5)	10.0	5.0
3	Pyrimethanil	17.43	198(100)	199(45)	200(5)	10.0	5.0
4	Propyzamide	18.94	173(100)	255(23)	240(9)	10.0	5.0
5	Pirimicarb	19.00	166(100)	238(23)	138(8)	20.0	10.0
6	Dimethenamid	19.77	154(100)	230(43)	203(21)	10.0	5.0
7	Tolclofos-methyl	19.83	265(100)	267(36)	250(10)	10.0	5.0
8	Fenchlorphos	19.90	285(100)	287(69)	270(6)	40.0	20.0
9	Pirimiphos-methyl	20.37	290(100)	276(86)	305(74)	20.0	10.0
10	2,4'-DDE	22.75	246(100)	318(34)	176(26)	25.0	12.5
11	Bromophos-ethyl	23.12	359(100)	303(77)	357(74)	10.0	5.0
12	4,4'-DDE	23.95	318(100)	316(80)	246(139)	10.0	5.0
13	Procymidone	24.57	283(100)	285(70)	255(15)	10.0	5.0
14	Picoxystrobin	24.79	335(100)	303(43)	367(9)	20.0	10.0
15	Chlorfenapyr	27.40	247(100)	328(54)	408(51)	200.0	100.0
16	Quinoxyfen	27.15	237(100)	272(37)	307(29)	10.0	5.0
17	Benalaxyl	27.68	148(100)	206(32)	325(8)	10.0	5.0
18	Bifenthrin	28.62	181(100)	166(32)	165(35)	10.0	5.0
19	Diflufenican	28.73	266(100)	394(25)	267(14)	10.0	5.0
20	Bromopropylate	29.46	341(100)	183(54)	339(51)	20.0	10.0

Table 2014.09B. SIM acquisition parameters by GC-MS for the 20 pesticides of interest in this study

Group	Start time, min	Monitored ions, *m/z*	Dwell time, ms
1	14.85	306,264,335	80
2	16.85	177,197,161,198,199,200	80
3	17.97	173,255,240,166,238,138	80
4	19.43	154,230,203,285,287,270,265,267,250	40
5	20.00	290,276,305,	80
6	21.77	246,318,176,353,355,351	80
7	22.93	359,303,357,318,316,246	80
8	24.20	335,303,367,283,285,255	80
9	25.87	237,272,307,247,328,408,148,206,325	40
10	28.49	181,166,165,266,394,267,341,183,339	40

Table 2014.09C. GC-MS/MS retention times, monitored ion transitions, collision energies, LODs, and LOQs for the 20 pesticides

No.	Pesticide	Retention time, min	Quantifying precursor/ product ion transition	Qualifying precursor/ product ion transition	Collision energy, V	LOQ, µg/kg	LOD, µg/kg
ISTD	Heptachlor-epoxide	22.15	353/263	353/282	17;17		
1	Trifluralin	15.41	306/264	306/206	12;15	4.8	2.4
2	Tefluthrin	17.40	177/127	177/101	13;25	0.8	0.4
3	Pyrimethanil	17.42	200/199	183/102	10;30	6.0	3.0
4	Propyzamide	18.91	173/145	173/109	15;25	1.0	0.5
5	Pirimicarb	19.02	238/166	238/96	15;25	4.0	2.0
6	Dimethenamid	19.73	230/154	230/111	8;25	2.0	1.0
7	Fenchlorphos	19.83	287/272	287/242	15;25	16.0	8.0
8	Tolclofos-methyl	19.87	267/252	267/93	15;25	10.0	5.0
9	Pirimiphos-methyl	20.36	290/233	290/125	5;15	10.0	5.0
10	2,4'-DDE	22.79	318/248	318/246	15;15	6.0	3.0
11	Bromophos-ethyl	23.16	359/303	359/331	10;10	10.0	5.0
12	4,4'-DDE	23.90	318/248	318/246	25;25	4.0	2.0
13	Procymidone	24.70	283/96	283/255	10:10	2.0	1.0
14	Picoxystrobin	24.75	335/173	335/303	10;10	10.0	5.0
15	Quinoxyfen	27.18	237/208	237/182	25;25	80.0	40.0
16	Chlorfenapyr	27.37	408/59	408/363	15;5	140.0	70.0
17	Benalaxyl	27.66	148/105	148/79	15;25	2.0	1.0
18	Bifenthrin	28.63	181/166	181/165	10;5	10.0	5.0
19	Diflufenican	28.73	266/218	266/246	25;10	20.0	10.0
20	Bromopropylate	29.46	341/185	341/183	15;15	8.0	4.0

Table 2014.09D. SRM acquisition parameters by GC-MS/MS analysis for the 20 pesticides of interest

Group	Start time, min	Monitored ion transitions, *m/z*	Dwell time, ms
1	14.76	306/264,306/206	50
2	15.87	177/127,177/101,200/199,183/102	50
3	18.06	173/145,173/109,238/166,238/96	50
4	19.26	230/154,230/111,287/272,287/242,267/252,267/93	25
5	20.07	290/233,290/125	50
6	21.87	353/282,353/263	50
7	22.60	359/331,359/303,318/248,318/246	50
8	23.59	335/303,335/173,318/248,318/246,283/96,283/255	50
9	26.71	148/105,148/79,408/363,408/59, 237/208, 237/182	25
10	27.88	266/246,266/218,181/166,181/165	50
11	28.96	341/185,341/183	50

Table 2014.09E. Gradient conditions for LC-MS/MS analysis

Step	Time, min	Flow rate, µL/min	Mobile phase A (0.1% formic acid water), %	Mobile phase B (acetonitrile), %
0	0.00	400	99.0	1.0
1	3.00	400	70.0	30.0
2	6.00	400	60.0	40.0
3	9.00	400	60.0	40.0
4	15.00	400	40.0	60.0
5	19.00	400	1.0	99.0
6	23.00	400	1.0	99.0
7	23.01	400	99.0	1.0

Table 2014.09F. LC-MS/MS retention times, ion transitions, collision energies, LODs, and LOQs for the 20 pesticides ofinterest in this study

No.	Pesticide	Retention time, min	Quantifying precursor/ product ion transition	Qualifying precursor/product ion transition	Collision energy, V	Fragmentation, V	LOQ, µg/kg	LOD, µg/kg
ISTD	Chlorpyrifos-methyl	16.01	322.0/125.0	322.0/290.0	15;15	80		
1	Imidacloprid	3.81	256.1/209.1	256.1/175.1	10;10	80	22.0	11.0
2	Propoxur	5.89	210.1/111.0	210.1/168.1	10;5	80	24.4	12.2
3	Monolinuron	6.83	215.1/126.0	215.1/148.1	15;10	100	3.6	1.8
4	Clomazone	8.3	240.1/125.0	240.1/89.1	20;50	100	0.4	0.2
5	Ethoprophos	11.37	243.1/173.0	243.1/215.0	10;10	120	2.8	1.4
6	Triadimefon	11.64	294.2/69.0	294.2/197.1	20;15	100	7.9	3.9
7	Acetochlor	12.94	270.2/224.0	270.2/148.2	5;20	80	47.4	23.7
8	Flutolanil	13.25	324.2/262.1	324.2/282.1	20;10	120	1.1	0.6
9	Benalaxyl	14.40	326.2/148.1	326.2/294.0	15;5	120	1.2	0.6
10	Kresoxim-methyl	14.58	314.1/267	314.1/206.0	5;5	80	100.6	50.3
11	Picoxystrobin	14.99	368.1/145.0	368.1/205.0	20;5	80	8.4	4.2
12	Pirimiphos methyl	15.05	306.2/164.0	306.2/108.1	20;30	120	0.2	0.1
13	Diazinon	15.20	305.0/169.1	305.0/153.2	20;20	160	0.7	0.4
14	Bensulide	15,45	398.0/158.1	398.0/314.0	20;5	80	34.2	17.1
15	Quinoxyfen	16.60	308.0/197.0	308.0/272.0	35;35	180	153.4	76.7
16	Tebufenpyrad	16.82	334.3/147.0	334.3/117.1	25;40	160	0.3	0.1
17	Indoxacarb	16.76	528.0/150.0	528.0/218.0	20;20	120	7.5	3.8
18	Trifloxystrobin	16.82	409.3/186.1	409.3/206.2	15;10	120	2.0	1.0
19	Chlorpyrifos	17.65	350.0/198.0	350.0/97.0	20;35	100	53.8	26.9
20	Butralin	17.98	296.1/240.1	296.1/222.1	10;20	100	1.9	1.0

Table 2014.09G. SRM acquisition parameters by LC-MS/MS analysis for the 20 pesticides

Group	Start time, min	Monitored ion transitions, *m/z*	Dwell time, ms
1	0	256.1/209.1, 256.1/175.1, 210.1/111.0, 210.1/168.1, 240.1/125.0, 240.1/89.1, 243.1/173.0, 243.1/215.0, 294.2/69.0, 294.2/197.1,215.1 / 126.0 ; 215.1 / 148.1	30
2	12	270.2/224.0, 270.2/148.2, 306.2/164.0, 306.2/108.1, 324.2/262.1, 324.2/282.1, 326.2/148.1, 326.2/294.0, 305.0/169.1, 305.0/153.2, 314.1/267.0, 314.1/206.0, 322.0/125.0, 322.0/290.0, 368.1 / 145.0, 368.1/205.0, 398.0 / 158.1, 398.0 / 314.0	20
3	16.4	334.3/147.0, 334.3 / 117.1, 528.0 / 150.0, 528.0 / 218.0, 409.3/186.1, 409.3 / 206.2, 296.1 / 240.1, 296.1 / 222.1,350.0/198, 350.0/97.0,308.0 / 197.0 ; 308.0/272.0	25

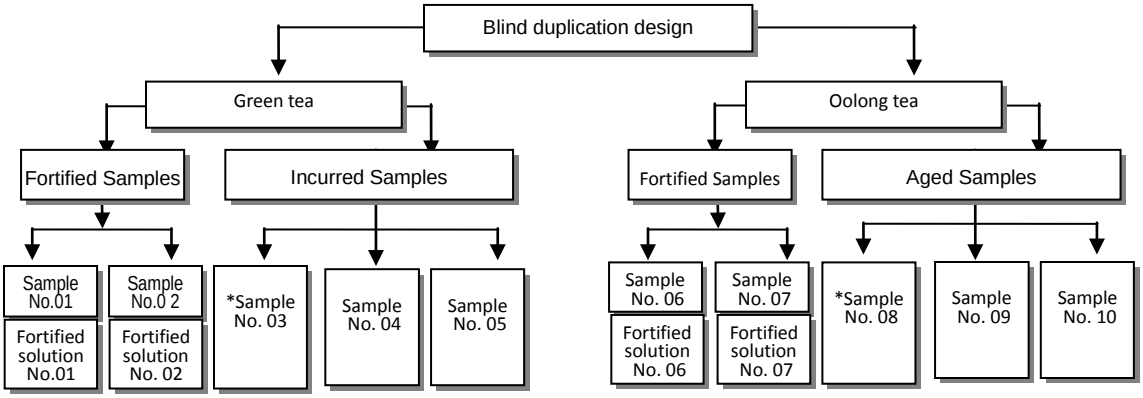


Figure 2014.09B. Number of blank, fortified, incurred, and aged samples for the collaborative study. Nos. 03 and 08 are unknown blank samples. Fortified samples are designed per principle of blinds duplication.

Table 2014.09H. Recommended maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques

Relative intensity (% of base peak), %	GC-MS (relative), %	GC-MS/MS, LC-MS/MS (relative), %
>50	±10	±20
>20-50	±15	±25
>10-20	±20	±30
≤10	±50	±50

Pesticide methods centric community to conduct the multi-laboratory collaborative study based on 20 selected compoounds that can be analyzed by GC-MS and 20 compounds that can be analysed by LC-MS/MS. Altogether, 560 samples covering the40 selected pesticides wereanalysed in the study. These samples included Green tea and Oolong tea samples fortified typically at the EU maximum residue limit (MRL) for regulatory guidance and compliance, aged tea samples incurred with 20 pesticides and Green tea and Oolong tea samples incurred with 5 pesticides. The analysis of the 560 samples generated a total of 82459 test results by the 30 participating laboratories. One laboratory failed to meet the proficiency requirements in the pre-collaborative study. Therefore, its data submitted for the collaborative study were excluded from further analysis and interpretation. The results presented are therefore the 6638 analytical results obtained from the 29 remaining laboratories. The 6638 target pesticide test data from the 29 remaining laboratoriesincluded 1977 results generated by GC-MS, 1704 results by GC-MS/MS and 2957 results by LC-MS/MS. It was determined after application of the Grubbs and Dixon tests for outliers to the data sets that there were 65 outlier results from the 1977 GC-MS results (3.3%), 65 outlier results from the 1704 GC-MS/MS results (3.8%) and 57 oultlier results out of 2957 results from the LC-MS/MS results (1.9%) representing 0.98, 0.98, and 0.86 %, respectively, of the 6638 results generated in the study.

Statistical analysis with the AOAC statistical software package also confirmed that AOAC OMA-2011-Jan-001Jan 001which had been validated under single laboratory conditions is rugged and the average recoveries, average concentration, RSD_r, RSD_R and HorRat values all meet the recovery and reproducibility criteria for use in multiple laboratories. The Study Director is strongly recommending this method for adoption as AOAC Official First/Final Action Method.

Introduction

Tea is considered one of the 3 most consumed beverages in the world and is enjoyed by over 2.0 billion people from more than 160 countries and regions in the world (1). It is reported that in 2011 alone, more than 50 countries around the globe grew tea, with tea plantation areas covering about 3.2 million hectares with an annual output of 4.7 million tons. China, India, Kenya, Sri-lankaand Turkey are the world's five largest tea producers, and their tea output makes up about 76% of the total world production (2). Tea grows mostly in warm temperate zones and subtropical regions and is subject to threats from diseases and pest infestations, so pesticides are widely used and hence the potential threat from pesticide residue

contamination. At present, 17 countries and international organizations including the Codex Alimentarius Commission, the European Union, Germany, Holland, Switzerland, Hungary, Israel, Italy, China, Japan, Korea, USA, Australia, India, Kenya, South Africa, etc. (till 2006) have established MRLs for over 800 pesticide residues (3). With the rapid awareness of consumers to the effect of food contamination on consumer health, an increasing demand to move to organic farming practices, the capability of today's analytical laboratories to detect lower and lower levels of contamination in foods, it is imperative that high throughput multi-class, multi-analyte methods be available to monitor residues of pesticides and other contaminants in a high-production food like tea. It is for this reason that this method was developed.

There are over 1000 listed pesticides and chemical contaminants used around the world for agricultural purposes. In the early 2000s , the author's team focused on the development of sensitive analytical methods and have published a series of papers (4-9) covering the study of the persistence of between 400 and 500 pesticide residues in 20 agricultural and fishery products including fruits and vegetables(10, 11), grains(12, 13), teas(14, 15), Chinese medicinal herbs(16, 17), edible fungi (mushrooms)(18, 19), animal tissues(20, 21), aquatic products(22, 23), raw milk and milk powders(24, 25), honey, fruit juices and fruit wines(26-28), potable water(29).

Since 2009, the authors have developed analytical methods for the determination and confirmation of 653 pesticide residues in tea and used the method to conduct some pivotal studies in an attempt to obtain a better understanding of the fate, distribution and persistence of pesticide residues in tea. The method was used to conduct:

(a) a 3-month stability study for 460 pesticides in six different types of solutions,

(b) a 3-month stability study for 345 pesticides in tea,

(c) a 3-month detection study on deviation ratios of 275 pesticides in Youden pairedtea samples,

(d) a 3-month study of the ruggedness of the method,

(e) a 3-month study on the degradation kinetics for 227 pesticides in aged tea samples,

(f) a 3-month verification study of the applicability of EU performance criteria standard (EU Document No.SANCO/10684/2009) (30) in an AOAC collaborative study, and

(g) a 3-monthexperimental field study on the degradation patterns and stability of pesticides in incurred tea samples.

Most recently (2011-2012), the method was used in a comparative study to evaluate the influence of tea

It will be unimaginably difficult in terms of resources, time and personnel for each collaborator to paticipate in a collaborative study on a method such as this one that covers residues of 653 pesticides and chemical contaminants. So, for this collaborative study an expert review panel within the AOAC International's Pesticide and Chemical Contaminants Method Centric Community 12 recommended a study protocol based on 20 representative compoounds that can be analysed by GC-MS and GC-MS/MS and 20 compounds that can be analysed by LC-MS/MS and applied to 2 teas for an inter-laboratory validation of this method in a collaborative study. The 40 pesticides recommended by the expert panel out of the 653 pesticides for this multi-laboratory validation study showing their EU MRLs are listed in Table 1.

Table 1 Pesticides selected for the Collaborative Study

No.	GC-MS&GC-MS/MS			LC-MS/MS		
	Pesticides	MRL/ (μg/kg)	Source	Pesticides	MRL/ (μg/kg)	Source
1	2,4'-DDE	200	EU	Acetochlor	10	EU
2	4,4'-DDE	200	EU	Benalaxyl	100	EU
3	Benalaxyl	100	EU	Bensulide	30	Japan
4	Bifenthrin	5000	EU	Butralin	20	EU
5	Bromophos-ethyl	100	EU	Chlorpyrifos	100	EU
6	Bromopropylate	100	EU	Clomazone	20	EU
7	Chlorfenapyr	50000	EU	Diazinon	20	EU
8	Diflufenican	50	EU	Ethoprophos	20	EU
9	Dimethenamid	20	EU	Flutolanil	50	EU
10	Fenchlorphos	100	EU	Imidacloprid	50	EU
11	Picoxystrobin	100	EU	Indoxacarb	50	EU
12	Pirimicarb	50	EU	Kresoxim-methyl	100	EU
13	Pirimiphos-methyl	50	EU	Monolinuron	50	EU
14	Procymidone	100	EU	Picoxystrobin	100	EU
15	Propyzamide	50	EU	Pirimiphos methyl	50	EU
16	Pyrimethanil	100	EU	Propoxur	100	EU
17	Quinoxyfen	50	EU	Quinoxyfen	50	EU
18	Tefluthrin	50	EU	Tebufenpyrad	100	EU
19	Tolclofos-methyl	100	EU	Triadimefon	200	EU
20	Trifluralin	100	EU	Trifloxystrobin	50	EU

(b) Concentration ranges for the analytes to be considered in the study

The concentration range selected for the 40 target pesticides is 10-2000 μg/kg based on guidance provided by an expert panel at two exclusive meetings organized by AOAC Pesticide and Chemical Contaminants Method Centric Community 12.One\ was at the Florida Pesticide Residue Workshop held July18-21, 2011 and the other was the 125th AOAC Annual Meeting held in New Orleans in September 2011.

(c) **Fortified and Blank Tea Samples to be Analysed for the Collaborative Study**

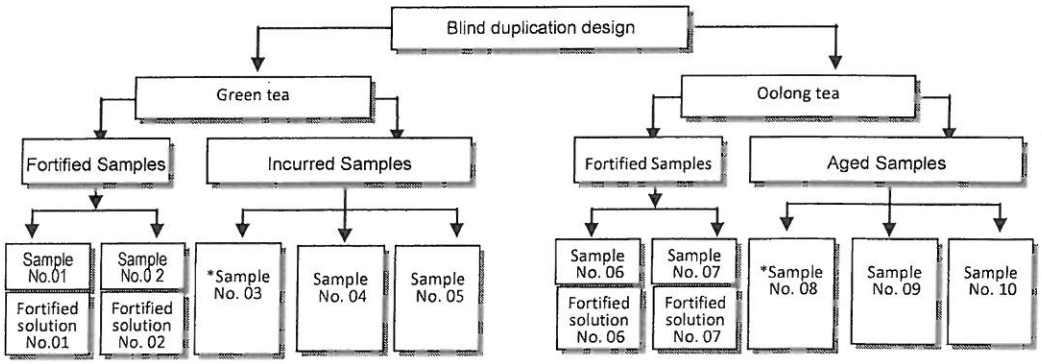
Every collaborator will be expected to analyse 4 Fortified Samples (Samples No.01 to No.02 for Green tea; No.06 to No.07 for Oolong Tea) and 2 Blank Samples.

(d) **Incurred Samples for Collaborative Study**

Four incurred samples [No.03 to No.05 for Green tea; No.08 to No.10 for Oolong tea] generated from a tea plantation named "Youshan Garden" in Fujian, where growing teas were sprayed with pesticides as per the relevant commercial procedure, picked at different phases of cultivation, dried and then processed will be tested as incurred samples.

(e) **Samples for pre-collaborative study**

To demonstrate proficiency at each participating laboratory, the SD will provide each collaborating laboratory with instructions on how to conduct the proficiency recovery test and at least five portions of 5 g blank Green tea and Oolong tea samples.Figure 1 below is a flow chart of the validation plan to be conducted for the collaborative study



* No.03 andNo.08 are unknown blank samples

Fig. 1 Number of blank, fortified, incurred and aged samples for the collaborative study

(Fortified samples are designed per principle of Blinds Duplication)

1.4 Quality assurance

1.4.1 Pre-collaborativestudy requirements

Prior to the determination of the collaborative samples, collaborators should practice testing the samples in advance in their own laboratories. No collaborator shall undertake the analysis of the official

collaborative samples until the SD has determined that satisfactory performance has been achieved for the pre-collaborative test samples. [Note: *Results from any laboratories that conduct the collaborative study without evidence of confirmation of proficiency status by the SD through a pre-collaborative sample analysis shall be automatically excluded from the analysis and interpretation of the collaborative study data].*

1.4.1.1 Pre-collaborative study experiments

Collaborators will conduct fortified recovery replicate tests with blank tea samples (Green tea or Oolong tea) and the mixed pesticide working standard solutions supplied and directed by the SD. To each 5 g blank tea sample add 50 μL mixed pesticide working standard solutions and conduct at least 5 replicate analysis. Calculate the analytical recovery, the% RSD (for n=5), the ion abundance ratio and the linear coefficient of determination (R^2) for the calibration curve and submitted tothe SD.

1.4.1.2 Pre-collaborative study qualification and acceptance criteria

To demonstrate proficiency in the use of the method, the laboratory results must satisfy the following acceptance criteria:

(a) R^2 for each of the 20 pesticides based on at least a 5 point matrix-matched calibration standard curve shall be greater than 0.995 for GC-MS, GC-MS/MS or LC-MS/MS;

(b) Recoveries shall fall within 70-120% with RSD<15% (n=5);

(c) The ion abundance of the targeted pesticides should be in accordance with recommended maximum permitted tolerances of the AOAC regulation or the EU regulation ["EU Document No. SANCO/10684/2009Method validation and quality control procedures for pesticide residues analysis in food and feed."And ISO 34]

[NOTE: If ⩾30% of the data generated in a laboratory for recovery, RSD, ion abundance or R^2 valuesfail the acceptance criteria, this laboratory will be considered non-proficient in the use of the method and the data generated from that laboratorywill beexcluded from the final analysis and interpretation of the data generated for the collaborative study]

1 **1.4.2 System suitability check**

2 Before making determinations of each consignment samples, use quality control standard working
3 solutions supplied by SD to check the sensitivity and stability of the instrument.
4 (1) Instrumental sensitivity (calculated per internal standard reference signal to noise ratios (S/N)): GC-
5 MS＞500, GC-MS/MS ＞1000, LC-MS/MS＞500.
6 (2) Instrumental stability (calculated per internal standard peaks): Retention time deviation<3% (2
7 successive injections); Peak area deviation <10% (2 successive injections).
8 (3) GC-MS, GC-MS/MS and LC-MS/MS must all be equipped with data processing software system.
9
10 **1.4.3 Reagent Blank and Pesticide residue-free (blank sample) test**
11 (1) Confirm there are no interference peaks from reagents by running a reagent blank test of the whole
12 process;
13 (2) This is done by running the reagent blank sample the same as described in the Collaborative Study
14 Method section 6 without adding the sample.
15 **1.4.4 Calibration check**
16 Prepare a 5-point matrix matched standard calibration curve with internal standard. The coefficient of
17 determination (R^2) for the calibration curve must be ≥0.995.
18
19 **2. AOAC Official MethodOMAMAN-14: High-throughput analytical techniques for the**
20 **determinationand confirmation of residues of 653 multi-class pesticides and chemical pollutants in**
21 **tea by GC-MS, GC-MS/MS and LC-MS/MS: A Multi-Laboratory Collaborative Study of AOAC**
22 **OMA-2011-Jan-001**
23 Forty representative pesticides were selected based on guidance from the Pesticide Method Centric
24 Community to conduct a multi-laboratory validation study of the single laboratory validated (SLV) method
25 OMA-2011-Jan-001 using 2 representative brands of tea. The representative pesticides selected for the
26 multi-laboratory validation study of the SLV method are as follows: 2,4'-DDE, 4,4'-DDE, Benalaxyl,
27 Bifenthrin, Bromophos-ethyl, Bromopropylate, Chlorfenapyr, Diflufenican, Dimethenamid, Fenchlorphos,
28 Picoxystrobin, Pirimicarb, Pirimiphos-methyl, Procymidone, Propyzamide, Pyrimethanil, Quinoxyfen,

2.6.2.4 Connect a 30 mL reservoir onto the upper part of the cartridge using an adapter (see Figure 3).

2.6.2.5 Elute the cartridge with 25 mL acetonitrile-toluene (3:1, v/v).

2.6.2.6 Evaporate the eluate to approx. 0.5 mL using a rotary evaporator (or TurboVap) in a 40 °C water bath.

For GC-MS and/or GC-MS/MS analysis ONLY:

2.6.2.7 Add 40 µL heptachlor epoxide (ISTD) working standard solution to the sample in step 2.6.2.6.

2.6.2.8 Evaporate to dryness under a stream of nitrogen in a 35 °C water bath (or Turbo Vap).

2.6.2.9 Dissolve the dried residue in 1.5 mL of hexane, ultrasonicate the samples to mix, filter through a 0.2 µm membrane filter. The sample is ready for GC-MS or GC-MS/MS analysis.

For LC-MS/MS analysis ONLY:

2.6.2.10 Add 40 µL chlorpyrifos methyl (ISTD) working standard solution to the sample prepared in step 2.6.2.6

2.6.2.11 Evaporate to dryness under a stream of nitrogen in a 35 °C water bath (or Turbo Vap).

2.6.2.12 Dissolve the dried residue in 1.5 mL of acetonitrile-water (3:2, v/v), ultrasonicate the samples to mix, and filter through a 0.2 µm membrane filter. The sample is ready for LC-MS/MS analysis.

2.7 Qualitative and quantitative analysis

2.7.1 Criteria for qualitative identification and confirmation.

2.7.1.1 Measure the retention time of the monitored peaks and match them with the same peaks on the pesticide standard chromatograms.

2.7.1.2 Measure the ion abundances for the qualifier ions for the detected pesticides and verify that they are within the expected limits; if the peaks match, the presence of the pesticide is confirmed. See Table 12.

Table 12. Recommended maximum permitted tolerances for relative ion intensities using a range of mass spectrometric techniques

Relative intensity (% of base peak)	GC-MS (relative)	GC-MS-MS, LC-MS/MS (relative)
>50%	±10%	±20%
>20%-50%	±15%	±25%
>10%-20%	±20%	±30%

instruments used in the analysis. It was these older generation instruments that the method generated significant variability in precision. Additionally, the concentration of 7 compounds added to Oolong tea were so low and so close to the lowest concentration point on the calibration curve thus contributing to the imprecision at the low end of the calibration curve. For example, this happened in the case of Lab 25 that determined that the measured concentration of these pesticides in the sample were lower than the minimum concentration point calculated concentration (Calc. Conc.) of matrix standard curve and did not report the result thus causing the quantification results at the low end of the calibration curves to show large and variable within-lab standard deviations. Some laboratories (e.g. Lab 21) did not report the test results when they found pesticide concentrations in the samples to be lower than the minimum concentration point of the calibration curves. In hind sight, the SD thinks they should have designed the experiments better to include a much lower minimum concentration point on the calibration curves (e.g. being 50% of fortification concentrations) than were designed in this collaborative study and they should have tried to ensure that the concentrations of pesticide residues in the samples after extraction were still above the minimum concentration points of calibration curves.

While these issues became apparent during the collaborative study, they were not seen as an issue during the single laboratory validation of the method where very good recoveries and precision were obtained for samples fortified at concentrations where the minimum point on the calibration curves used for quantification were 80% of the fortification concentrations of samples (see Table 19).Therefore, the SD hopes to pay more attention in any future organization of or participation in a collaborative study to this oversight and take appropriate measures to minimize its occurrence again in a future study design.

3.1.3 Method Extraction Efficiency and Reproducibility for Aged Samples

The method efficiency parameters such as Recovery, RSD_R, RSD_r and HorRat values in Table 20 are summarized in Table 21 per sectors.

3.1.3.1 By GC-MS

The results of the statistical analysis of data obtained from the analysis of the 20 pesticides in aged Oolong tea samples in Table 21 show that for the 16 collaborating laboratories using GC-MS, all the pesticides demonstrated within-lab repeatability $RSD_r < 8\%$, between-lab reproducibility, $RSD_R < 25\%$ and HorRat values less than 1.0.

3.1.3.2 By GC-MS/MS

For the 14 collaborating laboratories using GC-MS/MS, all the pesticides demonstrated within-lab repeatability, RSD_r < 15% whilst only 6 of the 20 (30%) pesticides demonstrated between-lab reproducibility RSD_R < 25%. Seventy percent (70%) of the 20 pesticides had between-laboratory reproducibility RSD_R>25%. The HorRat valuesfor all the pesticides were less than 2.0.

3.1.3.3 By LC-MS/MS

The within-laboratory repeatability RSD_r for the 24 laboratories using LC-MS/MS was<15% for all the pesticides, the between-lab reproducibility, RSD_R<25% for only 8 of the 20 pesticides. The other 12 (60%) pesticides showed between-lab reproducibility RSD_R>25%, The HorRat values were <2.0 for all 20 pesticides.

The probable expalanation for the large variability in RSD_R>25% for some of the pesticides in aged Oolong tea in the GC-MS/MS and LC-MS/MS analysis may be traced to just how aged samples are prepared. They are prepared by spraying pesticides onto dry tea powders in advance, which is then mixed uniformly. In a certain period after sample preparation, pesticides in tea slowly degrade during storage and transit, so in our single-laboratory validation at an earlier stage a two phase study was conducted to measure the rate of depreciation in concentration of pesticides with time and to determine the effect it has on the quantitative analysis of pesticides in tea samples. The studies described in ANNEX 1.5.4 concluded that pesticides will depreciate in concentration by approximately 4.3-31.1% over a 3-month period. Accordingly, correlation equations were derived for the depreciation rate in concentration andhavebeen defined for each of the pesticides (Figure 4) under study so thattheconcentration depreciation factor can be taken into consideration in the calculation of the concentration of a pesticide at any specified time during a study. On the basis of stability results of pesticides in aged Oolong tea determined by GC-MS/MS or LC-MS/MS in a continuous three month study (see ANNEX 1.5.4), trend charts were plotted in which, the determination time (day) was the X-axis and the difference between each concentration determined at time t and the concentration of pesticide estimated at time zero was the Y-axis. The logarithmic equations presented graphically in Figure 5 were obtained by fitting the three months determination results. From these equations, the degradation value of any of the 20 target pesticides at any specific day could be calculated and applied to the raw data generated for that pesticide in that particular laboratory.

logarithmic equation defined for each pesticide. To eliminate the influence from the natural degradation of pesticides, the rates of depreciation in concentration for each pesticide residue measured in each laboratory were pooled together into a database from which correlation equations were derived. The procedure was used to calculate corrected/revised method efficiency parameters as shown in Table 22 for Oolong tea. It can be seen from Table 22 that of the method performance parameters such as S_r, RSD_r, S_R, RSD_R and HorRat, only the RSD_R data are impacted most significantly by the degradation factor. So, only the raw data and corrected data of RSD_Ras shown in Table 23 would be discussed.

In the previous discussion, it had been noted that only 6 of the 20 pesticides analyzed by GC-MS/MS showed $RSD_R \leqslant 25\%$ while the other 14 pesticides had $RSD_R \geqslant 25\%$. Application of the depreciation correction factor to the previous data results in a revised $RSD_R$with the average of 22.8%, and the RSD_Rofthe remaining 14 pesticides with $RSD_R \leqslant 25\%$. A similar trend was observed for the 70% of the pesticides analysed by LC-MS/MS that showed $RSD_R > 25\%$.

In conclusion, it is the way the samples are prepared rather than any inherent weaknesses in the method that accounts for the unexpected large between-laboratory reproducibility data for some of the selected pesticides.

3.1.4 Method Extraction Efficiency and Reproducibility for Incurred Samples

The method efficiency parameters such as Rec., RSD_r, RSD_R and HorRat values in Table 24 are summarized in Table 25 per sectors.

3.1.4.1 By GC-MS

The statistical results of Green tea incurred samples in Table 25 show that concerning GC-MS: within-lab repeatability: ①$RSD_r < 8\%$ accounting for 100%, which illustrates that this method's repeatability is very good in different laboratories. ②between-lab reproducibility: $RSD_R < 25\%$ making up 100%, which demonstrates that the between-lab reproducibility is very good. ③HorRat values<1.0 accounting for 100%, which shows that the method has achieved very good reproducibility under the corresponding analytical conditions;

3.1.4.2 By GC-MS/MS

Concerning GC-MS/MS: ① within-lab repeatability: $RSD_r < 8\%$ accounting for 100%, which shows that the method's repeatability is very good in different laboratories. ②between-lab reproducibility: $RSD_R <$

25%making up 100%, which proves that the between-lab reproducibility is very good.③HorRat values<1.0 accounting for 100%, which reveals that the method has achieved very good reproducibility under the corresponding analytical concentrations;

3.1.4.3 By LC-MS/MS

Concerning LC-MS/MS: ①within-lab repeatability: RSD_r<15% accounting for 100%, which shows that the method's repeatability is very good in different laboratories. ②between-lab reproducibility: RSD_R<25% making up 100%.

To summarize the above-mentioned analysis, this collaborative study result shows that the method efficiency is acceptable.

3.2 Qualification and quantification

The collaborative study protocol stipulates that the deviations of qualifying ion abundance of the target pesticides shall have to comply with EU standards. For this collaborative study, GC-MS, GC-MS/MS and LC-MS/MS were used by 29 labs for the determination of pesticide residues in Green tea and Oolong tea (excluding blank samples), with 10231 target ion abundance ratio data obtained, and ion abundance ratios are checked item by item against EU standard confirmation criteria, with results tabulated in Tables 26-28.

3.2.1 Qualification of target pesticides

3.2.1.1 GC-MS

Tables 26-28 show that ~~concerning~~ GC-MS 16 labs participated in the study: a total of 4414 (2324+2090) target pesticide ions were obtained from determination of Green tea and Oolong tea samples, among which the ion abundance ratios that comply with EU standards reach 4154 (2229+1925), accounting for 94%.

3.2.1.2 GC-MS/MS

For GC-MS/MS, 14 laboratories participated in the study, a total of 2268 (1041+1227) target pesticide ions of Green tea and Oolong tea, among which the ion abundance ratios that comply with EU standards reach 2247 (1032+1215), accounting for 99%.

3.2.1.3 LC-MS/MS

For LC-MS/MS, 24 laboratories participated in the study, a total of 3549 (1819+1730) target pesticide ions of Green tea and Oolong tea samples, among which the ion abundance ratios that comply with EU

3.3 Error analysis and traceability

The 6638 effective data derived in this study are inspected with Grubbs and Dixon for outliers, with 187 outliers obtained, accounting for 2.8%. The distribution of outliers derived from these three methods GC-MS, GC-MS/MS and LC-MS/MS for different teas, different pesticide varieties and different samples are tabulated in Tables 30 to Table 32; The outliers from 10 samples of three categories determined by three different methods of GC-MS, GC-MS/MS and LC-MS/MS for two teas in Tables 30-32 are summarized and tabulated in Table 33.Distribution of outliers for different laboratories and different samples is listed in Table 34.

3.3.1 By GC-MS

Table 30, Table 33 and Table 34 show: each of 16 laboratories analyzed 20 pesticide residues in 8 samples (excluding 2 blank samples) and obtained 1977 effective data; the ~~inspection~~ of Grubbs and Dixon was adopted and 65 outliers ~~were discovered~~ making up 3.3%: they came from 11 laboratories, with 32 from Lab 19, accounting for 49.2% of the total outliers; outliers from other 10 laboratories are 33, totally make up 50.8%, and moreover outliers from each of these laboratories are less than 8, belonging to accidental deviations.

32 outliers from Lab 19 all came from No.4 and No.5 Green tea incurred samples and No.9 and No.10 Oolong tea aged samples. Test results from these four samples are 20-50% higher than those from other laboratories. Review of the experimental raw data record has found that this laboratory established the matrix calibration curves on Jun.8, and then the fortified sample of Green tea (No.1 and No.2) and Oolong tea (No.6 and No.7) were determimed, while No.4 and No.5 Green tea incurred samples and No.9 and No.10 aged samples were tested on Jun.12, with an interval of 4 days. Precise reasons that caused such deviations failed to be found in the raw data record, and it is assumed that during these 4 days interval instrument condition had changed possible, which may be the cause of such systematic deviations. For this, the collaborator considered that their instrument was stable since comparison of quality control sample had been conducted, but no reasonable explanation has been yet offered for the cause of such errors.

All in all, Lab 19 didn't carry out continuous inspection of samples after establishing calibration curves, with a time interval for four days before continuing inspection, which led to the results from their

To summarize the above-mentioned analysis, in the GC-MS/MS test results, overall deviations occurred with several specific samples for Lab. 21 and Lab. 18, resulting in two-thirds outliers concentrated in these 2 laboratories. Errors from other laboratories are accident deviations distributed in multi-kinds pesticides.

3.3.3 By LC-MS/MS

Tables 32-34 show: 2957 effective data were obtained from determination of 20 pesticide residues in 8 samples (excluding 2 blank samples) by 24 laboratories; Grubbs and Dixon inspection was adopted and 57 outliers ~~were discovered~~ (making up 1.9%). These 57 outliers came from 13 laboratories. There are 12 outliers from Lab.24, accounting for 21.1%; 11 from Lab 28, accounting for 19.3%; 9 from Lab 25, making up 15.8%, 6 from Lab 30, accounting for 10.5%. Outliers from other 9 laboratories total 19, accounting for 33.3%. Outliers from each of these 9 laboratories are less than 6, belonging to accidental errors.

11 of 12 outliers with Lab 24 from No.9 and No.10 Oolong tea age samples and the analytical results from these two samples are 40% lower than those from other laboratories. There are 11 outliers with Lab 28. These outliers came from 7 pesticides in 4 fortification samples of No.1, No.2, No.6 and No.7of 2 kinds of tea. It is mainly traced to accidental errors with determination of certain pesticides. There are 9 outliers with Lab 25. They came from 7 pesticides in 5 samples of No.1, No.2, No.6, No.7 and No.10 samples, belonging to accidental errors with certain pesticides. 6 outliers with Lab 30 came from Green tea fortification samples of No.1 and No.2, and the test results of Acetochlor, Benalaxyl, Bensulide, Kresoxim-methyl and Picoxystrobin in these two samples are about 40% greater than those from other laboratories, which also belong to accidental errors with certain pesticides.

To summarize the above-mentioned descriptions, there are 57 outliers from the 2957 effective data by LC-MS/MS, accounting for 1.9%. 57 outliers came from 13 laboratories, proving that they are relatively dispersed. No big systematic deviations happened with test results.

Expert Review Panel on Pesticide Residues
OFFICIAL CHAIR'S EXPERT REVIEW PANEL REPORT

ACKNOWLEDGMENT

The undersigned chair hereby confirms that the following document has been reviewed and constitutes the final version of the Official Chair's Report for the Expert Review Panel on Pesticide Residues held on Monday, September 8, 2014 during the AOAC Annual Meeting and Exposition at the Boca Raton Resort and Club, 501 East Camino Reel, Boca Raton, Florida 33432.

DR. JOE BOISON, CANADIAN FOOD INSPECTION AGENCY (CFIA)

Expert Review Panel Chair

October 14, 2014

Date

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EXPERT REVIEW PANEL MEETING ATTENDEES

Expert Review Panel Chair (s)
Joe Boison, Canadian Food Inspection Agency (CFIA)

Expert Review Panel Members
Amy Brown, Florida Department of Agriculture
Jo Marie Cook (Alternate), Florida Department of Agriculture
Julie Kowalski, Restek Corporation
John Reuther, Eurofins
Marina Torres, LATU
Jian Wang, Canadian Food Inspection Agency (CFIA)
Xiaoyan Wang, United Chemical Technologies, Inc. (UCT)

Method Authors
Dr. Guo-Fang Pang, Chinese Academy of Inspection and Quarantine

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Observers
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Tyson Friday, Frontier Coop
Jana Huckrabolt, Institute of Chemical Technology
Tom Phillips, MD Department of Agriculture
Jana Pugerhbovn, Institute of Chemical Technology
Nancy Thiex, AOAC RI Consultant
David Whitman, 3M Food Safety
David Woollard, Hill Laboratories (NZ)

EXPERT REVIEW PANEL, METHOD BACKGROUND, AND CONCLUSIONS

Criteria for Vetting Methods to be considered:
AOAC convened the *Official Methods of Analysis*^{SM} (OMA) Expert Review Panel for Pesticide Residues on Monday, September 8, 2014 from 4:30pm to 7:30pm during the AOAC Annual Meeting and Exposition in Boca Raton, Florida. The purpose of the meeting was to 1) Review the Collaborative Study Manuscript/ OMAMAN-14: High-Throughput Analytical Techniques For The Determination And Confirmation Of Residues Of 653 Multi-Class Pesticides And Chemical Pollutants In Tea By GC-MS, GC-MS/MS And LC-MS/MS (Study Director: Guo-Fang Pang, Chun-Lin Fan,Yan-Zhong Cao, Fang Yang, Yan Li, Jian Kang, Hui Chen, Qiao-Ying Chang, Chinese Academy of Inspection and Quarantine, No. 3 Gaobeidian North Rd 100123, Chaoyang District, Beijing, People's Republic of China) and to 2) discuss First to Final Action requirements and feedback mechanisms. The candidate method was reviewed against the approved collaborative study protocol. Supplemental information was also provided to the reviewers which included the collaborative study manuscript, Appendix 1.1-1.5 Analytical Instrumentation used in the Collaborative Study, Appendix 2 Determination Results of Collaborative Study, Appendix 3: Results of Practice Samples, Appendix 4: The Statistical Results, and an Journal Article: High-Throughput GC-MS and HPLC-MS/MS Techniques for the Multiclass, multiresidue Determination of 653 Pesticides and Chemical Pollutants in Tea.

Criteria for Vetting Experts and Selection Process:
The following seven (7) candidates and one (1) alternate member were submitted for consideration by the Official Methods Board to evaluate candidate methods for Pesticide Residues methods as per the Expert Review Panel (ERP) Policies and Procedures. The candidates were highly recommended by the Chemical Contaminants and Residues in Foods Community, have participated in various AOAC activities, including but limited to, Method Centric Committees that were formed under the legacy OMA pathway, and were vetted by the Official Methods Board. The experts are Amy Brown, Jo Marie Cook (Alternate), Julie Kowalski, John Reuther, Marina Torres, Jian Wang, and Xiaoyan Wang.

ERP Orientation:
The ERP members have completed the mandatory AOAC Expert Review Panel Orientation Webinar on Wednesday, July 16, 2014.

Expert Review Panel Meeting Quorum
The meeting of the Expert Review Panel was held in person. A quorum is the presence of seven (7) members or 2/3 of the total vetted ERP, whichever is greater. Seven (7) out of the seven (7) voting members were present and therefore met a quorum to conduct the meeting.

Standard Method Performance Requirements (SMPRs): N/A

Conclusion:
The Expert Review Panel reviewed OMAMAN-14: High-Throughput Analytical Techniques For The Determination And Confirmation Of Residues Of 653 Multi-Class Pesticides And Chemical Pollutants In Tea By GC-MS, GC-MS/MS And LC-MS/MS and adopted this method for First Action Official Method status by a unanimous decision with additional revisions as noted in the meeting minutes.

Subsequent ERP Activities:
ERP members will continue to evaluate the method for 2 years.

MEETING MINUTES

I. Welcome and Introductions

The Expert Review Panel Chair, Dr. Joe Boison welcomed Expert Review Panel members and initiated introductions. The Chair discussed with the panel the goal of the meeting.

II. Review of AOAC Volunteer Policies

Deborah McKenzie presented an overview of AOAC Volunteer Policies, Volunteer Acceptance Agreement and and Expert Review Panel Policies and Procedures which included Volunteer Conflicts of Interest, Policy on the Use of the Association, Name, Initials, Identifying Insignia, Letterhead, and Business Cards, Antitrust Policy Statement and Guidelines, and the Volunteer Acceptance Form (VAF). All members of the ERP were required to submit and sign the Volunteer Acceptance Form.

III. Expert Review Panel Process Overview and Guidelines

Deborah McKenzie presented an overview of the Expert Review panel process. The presentation included information regarding method submission, recruitment of ERP members, composition and vetting expertise, method assignments, meeting logistics, consensus, First Action to Final Action requirements, method modifications, publications, and documentation.

IV. Review of Methods

All ERP members presented a review and discussed the proposed collaborative study manuscript for High-Throughput Analytical Techniques for the Determination and Confirmation of Residues of 653 Multi-Class Pesticides and Chemical Pollutants in Tea by GC-MS, GC-MS/MS and LC-MS/MS. The method author, Dr. Guo-Fang Pang of the Chinese Academy of Inspection and Quarantine, was present and able to address questions and concerns of the ERP members. A summary of comments was provided to the ERP members.[1]

MOTION:
Motion by Wang, J.; Second by Reuther that this method be recommended for First Action Official Method Status.
Consensus demonstrated by: 7 in favor, 0 opposed, and 0 abstentions (Unanimous). *Motion Passed.*

MOTION:
Motion by Wang, J.; Second by Brown that the revisions requested by the ERP be provided for review.

- **Provide data on the parameters of the method for all of the 653 Analytes**
- **Provide clarity to the text**
- **Include the data on hydration**

Consensus demonstrated by: 7 in favor, 0 opposed, and 0 abstentions (Unanimous). *Motion Passed.*

V. Discuss Final Action Requirements for First Action Official Methods (if applicable)

No further action was discussed at this time.

VI. Adjournment

[1] Attachment 1: Summary of Expert Reviewer Comments for OMAMAN-14

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 Formula, from the National Institute of Standards and Technology (Gaithersburg, MD, USA) with a reference value for vitamin B_{12}.

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

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, USA) or Stuart LB3 (Barloworld, Bibby Sterilin Ltd, Staffordshire, UK).

(**g**) *Heating block*.—With nitrogen evaporation (Pierce Biotechnology, Inc., Rockford, IL, USA).

(**h**) *Vortex*.—Scientific Industries, Inc. (Bohemia, NY, USA).

(**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, USA).

(**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, USA) 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.

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(**d**) *Milli-Q water*.—Millipore (Bedford, MA, USA). 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, USA).

(**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).

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

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(**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:

$$\frac{(\boldsymbol{A-I})\times \boldsymbol{V_0}\times \boldsymbol{V_2}\times \mathbf{100}}{\boldsymbol{S}\times \boldsymbol{m}\times \boldsymbol{V_1}\times \mathbf{1000}}$$

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.

(**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.

References: Campos-Giménez, E., Fontannaz, P., Trisconi, M.J., Kilinc, T., Gimenez, C., & Andrieux, P. (2012) *J. AOAC Int*. **95**, 307–312 DOI: 10.5740/jaoacint.CS201108

J. AOAC Int. **97**, 1397(2014) DOI: 10.5740/jaoacint.14-119

AOAC SMPR 2011.05 *J. AOAC Int*. **95**, 293(2012) DOI: 10.5740/jaoac.int.11-0441

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Candidates for 2016 Method of the Year

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

Received March 29, 2014.
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.
Corresponding author's e-mail: esther.campos-gimenez@rdls.nestle.com
An appendix is available on the *J. AOAC Int.* website at http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac
DOI:10.5740/jaoacint.14-119

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

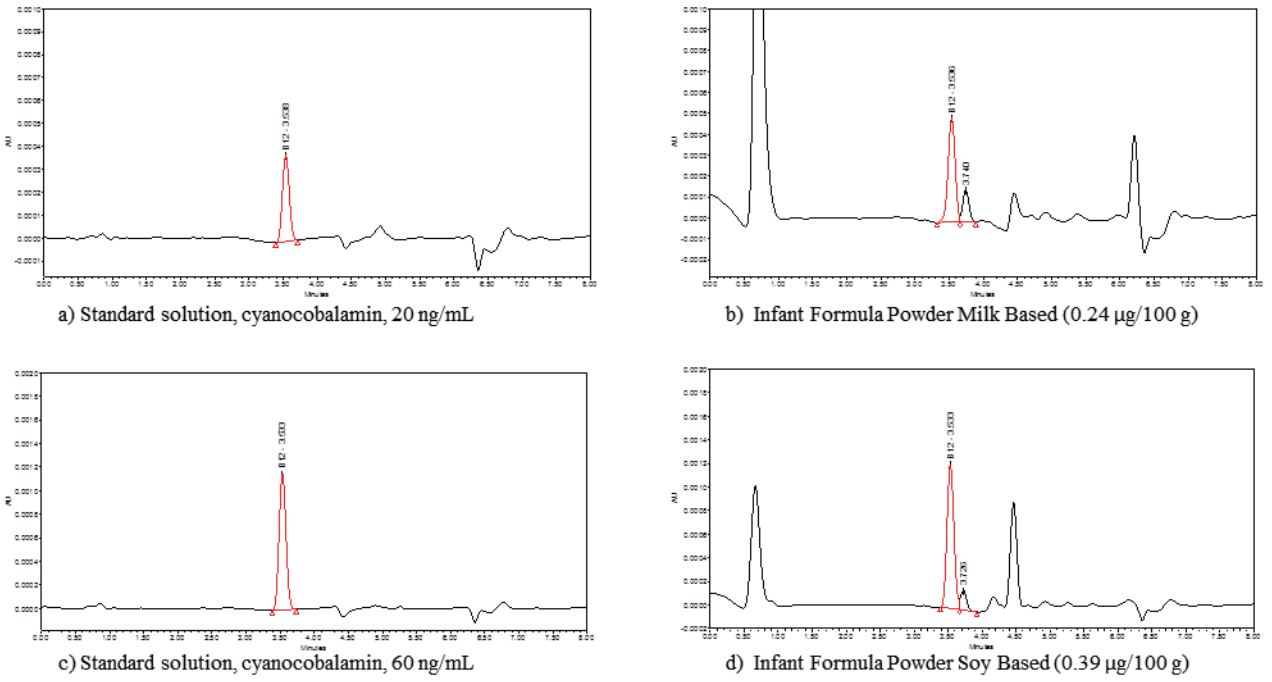


Figure 1. Example chromatograms of standard solutions at 20 (a) and 60 ng/mL (c) and infant formula powders (b, d).

(**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:

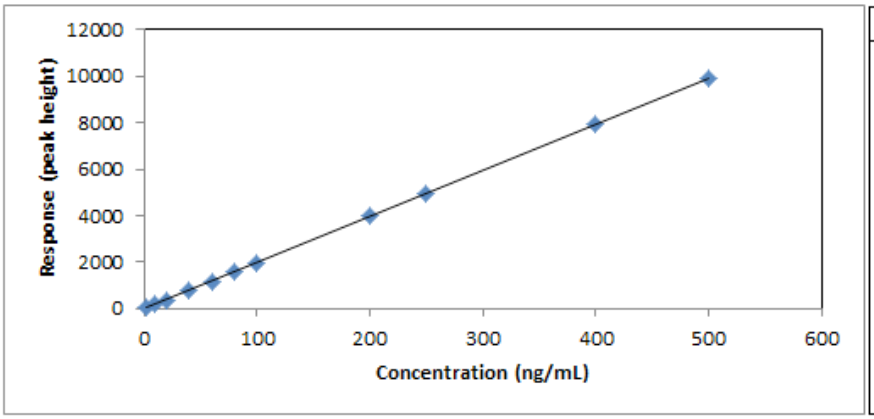
$$\text{Recovery (\%)} = \frac{C_{spiked}- C_{native}}{C_{added}} \times 100$$

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.

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(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

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 liquid chromatography with tandem mass spectrometry (HILIC LC-MS/MS).

B. Apparatus

(**a**) *Column.*--Acquity UPLC BEH HILIC 1.7 µm, 2.1 × 100 mm (Waters Corp., Milford, MA) or equivalent.

(**b**) *Liquid chromatograph.*—Waters Acquity UPLC Binary.

(**c**) *Solvent manager.*--Capable of 15,000 psi or equivalent (Waters Corp.).

(**d**) *Detector.*—Waters Acquity TQD or Xevo TQ-S 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.*–MarsXpress (CEM, Matthews, NC) or equivalent.

(**j**) *Reaction vessel.*--50 mL Teflon® (CEM, Matthews, NC, or equivalent).

(**k**) *Centrifuge tubes.*—Polypropylene (PP), 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, and 250 mL.

C. Reagents (Solvents and Chemicals)

(**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, Merck, Darmstadt, Germany, or equivalent).

(**f**) *pH buffer solutions*.--pH 4.0, 7.0, and 10.0.

D. Standards

(**a**) *L-Carnitine*.--USP Reference Standard Cat. No. 1359903; 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.

(**d**) *Choline chloride-d_9 (trimethyl-d_9 98%)*.--DLM-549-1 (Cambridge Isotope Laboratories Inc.).

E. Solution Preparation

(**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 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.

(*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 (MIX-IS) solution*.--Prepare fresh on day of analysis. Must use same MIX-IS solution for both calibration working standards (WS) 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**) *WS solution.*–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 solutions and in the three WS solutions.

F. Procedure

(**a**) 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**) 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 about 8 mL.

(*4*) Add 2.2 mL of about 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 around 3.5 N.

(*5*) Vortex vessels for 30 s, and then place vessels into turntable and insert into microwave.

(*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 into acetonitrile are stable for 24 h 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 MIX-IS, and dilute to 10 mL in a tube. Mix and then dilute this solution 1 to 25 mL with water in another tube. Mix, filter about 1 mL of sample slurry, and then dilute 10x with acetonitrile as above. No nitric acid or ammonia is used.

G. Instrument Operating Conditions

(**a**) *Mass spectrometry 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 additives (recommended by column supplier). After verifying equilibration of the UPLC system, inject the mid-level WS four times to verify system suitability. %RSD of the peak areas from these injections should be <5%. Once system suitability has been established, inject WS 1-3, followed by a reagent blank, control sample, and samples. Reinject WSs 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 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 WSs, the software plots each relative response (analyte/IS) versus its corresponding WS 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/IS) for choline and carnitine and uses the linear regression equation to calculate the resulting concentration in analytical solution.

(**c**) The concentration in the analytical solution (ng/mL) is multiplied by a dilution factor to project the results back to the original sample, on a µg/g basis:

$$C_x = C_s \times DF \quad \text{(Equation 1)}$$

where C_x is the concentration of the analyte in the product (µg/g), C_s is the concentration in the analytical solution measured by the instrument (ng/mL), and DF is the general dilution factor.

(**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/sample weight (g) (Equation 2)

(**e**) Thus, the final result (C_x) can be calculated by combining Equations 1 and 2.

Notes: (*1*) Free choline or carnitine can be determined by weighing same sample size (as for microwave digest procedure) into a disposable tube, skipping the microwave digestion and filtering the sample at an equivalent dilution (neither ammonia nor nitric acid to be added).

(*2*) Unless the sample contains milk proteins, free carnitine = total carnitine (through microwave digestion). Thus, the method is able to determine free and total carnitine and total choline simultaneously through microwave digestion. The nonmicrowave preparation is typically used for free choline in products (rare) or to determine free choline/carnitine in premixes. Note that the typical control sample, SRM 1849a, has substantially different values for carnitine depending on whether the SRM is microwave digested.

References: *J. AOAC Int*. (future issue)

AOAC SMPR 2012.010
J. AOAC Int. **96**, 488(2013); DOI: 10.5740/jaoac.int.SMPR2012.010

AOAC SMPR 2012.013
J. AOAC Int. **96**, 492(2013); DOI: 10.5740/jaoac.int.SMPR2012.013

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Table 2014.04A. Volumes of each stock solution for IWS			
IWS	Stock volume, µL	MIX-IS, µL	Final volume, mL
1	25	100	50
2	125	100	50
3	250	100	50

Table 2014.04B. Concentration of standards						
Standard	Choline	Choline chloride-d_9	Carnitine	Carnitine chloride-d_3	Diluent	Final volume, mL
	µg/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	10
WS 2	150	32	37.5	16	MeCN	10
WS 3	300	32	75	16	MeCN	10

Table 2014.04C. Microwave digestion parameters	
Power	100% (1600 W)
Ramp to temperature	10 min
Hold time	40 min
Temperature	120°C

Table 2014.04D. Mass analysis parameters (Xevo TQ-S)						
Standard	Retention time typical, min	Molecular ion (precursor)	Product ion	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	2.0 kV
Collision gas pressure	2–5 × 10^{-3} mtorr
Source temperature	150°C
Source offset	30 V
Desolvation temperature	550°C
Cone gas flow	300 L/h
Desolvation flow rate	1000 L/h
Peak width at half height	0.7 amu[a]
[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°C
Injection volume	10 µL
Injection type	Full loop
Sample temperature	8°C

Table 2014.04G. Gradient profile				
Time, min	Flow, mL/min	Mobile phase A, %	Mobile phase 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

Single Laboratory Validation of CARN-05: Determination of Free and Total Carnitine and Choline in Infant Formulas and Adult Nutritional Products

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July 25, 2014

Summary

A single laboratory validation (SLV) has been performed for a method that simultaneously determines choline and carnitine in nutritional products by LC-MS/MS. All eleven SPIFAN matrices (from the AOAC Stakeholder Panel on Infant Formulas and Adult Nutritionals) were tested. Depending on the sample preparation, either the added (free, with a water dilution and filtering) or total (after microwave digestion at 120° in nitric acid and subsequent neutralization with ammonia) species can be detected. For non-milk 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 (SMPRs) for carnitine and choline have been met. This document summarizes the material sent to the SPIFAN ERP (Expert Review Panel) for the review of this method. The purpose of performing the SLV is to enlist this method as a candidate for AOAC First Action Status for carnitine, and also choline as well.

Background

Over the last three years, as part of the SPIFAN program, the AOAC has issued regular Calls For Methods to find suitable test methods for global dispute resolution purposes for nutritional products. Choline was considered earlier in the process. SMPR 2012.013 was written and approved in 2012 (in February, India), describing the performance requirements needed for a choline dispute-resolution method. Three methods were chosen for consideration from those submitted. Abbott Nutrition submitted CHOL-06 (OMA #2012.19), an LC-MS/MS method very similar to the present submission. A little later, in September 2012, SMPR 2012.010 was written and approved for carnitine. Abbott submitted CARN-01 (AOAC 2012.17) in response to the Call For Methods, and this was the only entry for some time.

In 2013, the original Abbott choline method 2012.19 was revamped to meet internal requirements and to make logical improvements to it. Carnitine was added as an analyte (similar to Nestlé's publication in JAOAC 91, 4, 2008) and microwave digestion was added to speed up the typical 3-hour digestion (similar to NIST publication JAOAC 95, 5, 2012, p. 1479). The combined method was substantially different than either 2012.19 or 2012.17, and so both methods were withdrawn from the SPIFAN process. Since the last data was presented to the ERP in March 2014, the method has been put in final

form. The microwave digestion volume is now 10 mL instead of 20 mL. Also, the data presented in March only represented 3 independent analyses of the SPIFAN materials. The present submission CARN-05 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 is also presented for choline in case the ERP also wants to advance it for this use.

Method Outline

Below is an outline of the method. A more complete version is appended.

1. Weigh RTF or recon powder (1-5g) into microwave vessel.
2. Add 125 µL internal standards (d_3 -carnitine and d_9-choline) and water to 15 mL total volume (if only free determined; however, if Total is needed add only 10 mL water as the additional 5 mL will come in steps 3 and 4). Proceed to Step 5 for Free analysis (no ammonia or acid added). DF at this stage (using typical 3g sample size), is 15 mL/3.0g = 5.0
3. Total: Add 2.2 mL 70% HNO_3, seal, digest for 40 minutes at 120°C. Cool.
4. Add 3.5 mL 30% ammonium hydroxide to each vessel. Mix and stand for 30 minutes.
5. Add 1 mL of above solution to 25 mL water in disposable tube, mix, and filter with 0.45µ Nylon syringe filter. (25x dilution)
6. Makeup 0.1 mL of above filtrate with 0.9 mL ACN and inject. (10x dilution)
7. Simultaneous choline/carnitine on BEH HILIC column
8. Standards are nominally 30, 150, and 300 ppb choline (as hydroxide) and 8.0, 40, and 80 ppb carnitine with about 30 ppb choline-d9 internal standard and about 15 ppb carnitine-d3 IS.
9. Total dilution is approximately 5 x 25 x 10 = 1250, but depends on sample weight

Features

- Carnitine is the subject of this SLV, but total choline is also obtained simultaneously with proven accuracy
- Time to Result: < 8 hours possible, but in most cases LC-MS/MS runs overnight
- Advantages of internal standard, added up front for good precision
- HILIC column compatible with many methods (e.g. melamine)
- Validated on two substantially different Waters models of LC MS/MS. Note that other similar publications had used single quad MS.

System Suitability

- Calibration residuals <4%
- Internal Standard recovery 75-125% required (exact range TBD)
- Confirmatory transition required (ratio limits relative to standard TBD)
- Control sample SRM 1849a must be within limits

Discussion of Data

An amended SMPR Evaluation Form has been submitted to the ERP, containing all the SLV data for carnitine and choline. Tab 1, Method Evaluation Form, is filled out at the top with the SMPR requirements (from the website) and the CARN-05 performance summary. In LOD/LOQ section, note that we have chosen to spike Adult Milk Powder, 11750017V3, at the required LOQ limits in the SMPR, rather than supply the lowest limit we can actually quantitate. This is because we had some difficulty in pinning down the LOQ. Measuring the blanks, which were essentially pure water, with some purified ammonium nitrate after the digestion, provided very erratic baseline signals, especially in comparing the two instruments used. Therefore, multiplying this noise by 10 to calculate the LOQ seemed speculative. The more informative approach was to spike a product matrix at the LOQ limit expressed in terms of solution concentration:

For choline, the SMPR requirement is LOQ=2.0 mg/100g RTF. That's 20 ppm or 20,000 ppb. With a dilution factor of roughly 1000x for a 3g sample (see method outline above), the quantitation of a 20 µg/L addition of choline in solution is needed.

For carnitine, the SMPR says LOQ = 0.16 mg/100g RTF. That's 1.6 ppm, or 1600 ppb. Applying DF for a 3g sample, we must be able to quantitate 1.6 ppb in solution (we chose 1.0 ppb to be conservative).

Table 1, Tab 2, shows the spike recovery data on each matrix. This was performed in duplicate on each of three days (duplicate spikes as well as duplicate baseline values which were averaged). The spiking was carried out at two levels: an additional 40% and 80% of the native level. This caused us to have to extend the calibration curve a bit to accommodate the spiked samples that would normally be outside the calibration range. Choline and carnitine were spiked together in one set of spiking experiments, then acetylcarnitine was spiked separately in another 3 days of experiments. For a couple of products, the native level for carnitine and acetylcarnitine is shown in red (zero level) because we spiked into these Adult Powder products at a more routine level to demonstrate normal recoveries – the level was not 40% or 80% of the native level. Note that we later reduced the spike level into 11750017V3 to demonstrate the LOQ for carnitine – this matrix proved to be an adequate placebo for carnitine. This powder was also low in choline. We had to weigh an abnormally large sample size of 7.5g to try to adequately measure the choline and carnitine in the unspiked sample, which may explain the poor recovery (shown in red) for 11750017V3 at the 40% level. When the sample size was brought down to a more reasonable level of 3.0g, the choline recovery was very good (see Table 5b and LOQ data).

Tables 2a and 2b, Tabs 3&4, show the means and SD of nine independent days of SRM 1849a analyses. The total choline mean was 94.2% of the certified mean and the total carnitine mean was 110.3% of the certified mean. The NIST values tab, Tab 5, shows that Abbott values were almost identical to those obtained by the NIST lab themselves, with their very similar LC-MS method (publication referenced earlier). The NIST certificate labels the result "free" carnitine, but it is in fact determined after a microwave digestion and should be compared to Abbott's "total" carnitine result. The true free carnitine is about 88% of the total in SRM 1849a (see Table 7d). Perhaps there was some confusion in the

collection of other laboratories' data for the Certificate, as the Certified value is closer to the free carnitine than to the total carnitine.

Tables 3a and 3b, Tabs 6&7, show the Repeatability statistics for Total and Free determinations, respectively. These tabs also include Intermediate Precision data. In our laboratory, our SLV was carried out as follows. On Day 1, Analyst 1 prepared duplicate free and duplicate total preparations of each matrix and then split them into two portions. Half were put on Instrument 1 (a Waters TQD) and half were put on Instrument 2 (a Waters TQMS). The samples were analyzed overnight. On Day 2, Analyst 2 repeated this procedure, and so on, until each Analyst had collected 3 Days of data. Thus, there are 6 days of independent preparations in this protocol, but additional information was collected as far as how two different instruments' results agreed (from the split samples, which were not independent). The data shown in Tables 3a and 3b represent Days 1-3 from Instrument 1, and Days 4-6 from Instrument 2. This kept all data independent while maximizing the sources of variance from different days, analysts, and instruments. From the data on duplicates collected each day, ANOVA extracted the repeatability data that is shown in Tables 3a and 3b, while the overall RSD for the six days represents the intermediate precision, which is also listed. The Repeatability RSD for choline and carnitine were all in the 2% range or lower, easily meeting the SMPR requirements of 5% for choline and 8.0% for carnitine. The intermediate precision RSDs were mostly in the 2-3% range, which is a good indication that the method will meet the required Reproducibility of 10% for choline and 15% for carnitine. Note that the free choline level in Adult Milk Powder (0.36 mg/100g RTF, Table 3b) appears to be right around the LOQ because the short term precision is fine, but the intermediate precision begins to degrade. This level is about 10x lower than the required LOQ of 2 mg/100g from the SMPR, a level at which we obtained good recovery (Table 5b).

Table 4, Reproducibility, Tab 8, is empty because these data have not been collected yet. They would be collected during a MLT study, if the method is selected to proceed to such.

Table 5a (Tab 9), Linearity, gives some information about the calibration standards, sample sizes, dilution factors, and the curve linearity. To demonstrate linearity, the normal calibration curve was made, and then calibration standards were run as samples against this curve to determine the calibration bias (residuals) at each level. The average recovery over 3 days for carnitine at the 3.4 µg/L level (50% of the normal WS1 level) was 101.2%; for choline at the WS1 level of 30 ppb, it was 100.3%. These residuals at low levels reflect the excellent linearity of the curve (usually r = 0.9999) and the low blank values.

Table 5b, Tab 10, shows the results of spiking the Adult Milk Powder at a level that corresponds to the LOQ required by the SMPRs of choline and carnitine. The spike recovery of choline (96.9%, 0.9% RSD for seven replicates in one day) and carnitine (105.9%, 1.4 %RSD) indicate no problem with determining these low amounts in a product matrix. The Adult Milk Powder product had virtually no inherent carnitine and was therefore a good placebo for it. As shown in table, after spiking this product measured 0.17 mg/100g with a 1.5% RSD, indicating that there was no problem with the determination at the required LOQ.

Table 6, Tab 11, shows the Abbott SLV results for choline compared to the Thermo Fisher ion chromatography method (this first action method is currently being tested for Reproducibility in an MLT Study), AOAC 999.14 (the legacy colorimetric method, with enzyme treatment, conducted on our behalf by Covance Labs, 2 days in duplicate means), and the legacy Abbott method that employs an immobilized enzyme reactor (IMER) to convert choline to betaine and peroxide. The latter method detects the concentration of peroxide with electrochemical detection, which is proportional to the choline concentration in the sample.

Table 7a-d, Tab 12, shows the 6 x 2 means for each matrix as a function of instrument for choline and carnitine, and the overall 6x2x2 means across both instruments. It also compares the free vs. total concentrations.

The final tab, Tab 13, lists the previous ERP comments and how they were handled for this SLV.

Following is a more detailed summary of method equipment and procedures.

Simultaneous Determination of Choline/Carnitine in Infant Formulas and Adult Nutritional Products by HILIC LC-MS/MS

Wei Jing, Lou Salvati, and Joseph J. Thompson, Abbott Nutrition, Columbus, Ohio USA

Principle

The 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 liquid chromatography with tandem mass spectrometry (HILIC LC-MS/MS).

A. Apparatus
   a. *Column* - Acquity UPLC BEH HILIC 1.7 µm, 2.1×100 mm (Waters Corp., Milford, MA) or equivalent
   b. *Liquid chromatograph* —Waters Acquity UPLC Binary
   c. *Solvent Manager* - capable of 15 000 psi or equivalent
   d. *Detector* —Waters Quattro Premier XE, or Xevo TQ-S triple quadrupole mass spectrometer, or equivalent
   e. *Injector* —Waters Acquity sample manager with integrated column oven or equivalent
   f. *Nitrogen generator*.—Peak Scientific (Billerica, MA) Model NM30LA or equivalent
   g. *Data system* —Waters MassLynx, latest revision or equivalent
   h. *Microwave* – MarsXpress (CEM, Matthews, NC) or equivalent
   i. *Reaction Vessel* - 50 mL TEFL reaction vessels (CEM, Matthews, NC)
   j. *Centrifuge tubes* — PP (Polypropylene), 50 mL capacity disposable or equivalent
   k. *Vortex mixer with flat and cone top*
   l. *Balance1* — Readable to at least 0.0001 g. (Mettler-Toledo AT200 or equivalent)
   m. *Balance2* — Readable to at least 0.001mg. (Mettler-Toledo XP 6 or equivalent)
   n. *Syringe* – 1 mL Luer tip
   o. *Filters* – 0.45 µm Nylon membrane with syringe tip or equivalent
   p. *Pipets* - 5~25, 50~250, and 100~1000 µL adjustable
   q. *Pipet tips*

B. Reagents
   a. Solvents and Chemicals
      i. *Acetonitrile* —Fisher Optimal LCMS grade, or equivalent
      ii. *Nitric Acid* — 69-70% Reagent grade. J.T. Baker or equivalent
      iii. *Ammonium Acetate* —Fluka LCMS additive grade or equivalent
      iv. *Ammonium Hydroxide* —28 – 30% Reagent grade (for adjusting pH in mobile phase also, check it before use). MACRON or equivalent.
      v. *Distilled or deionized laboratory water* —Purified water, USP; ≥18Mohm/cm.

C. Standards
   a. *L-Carnitine*, USP Reference Standard Cat. No. 1359903, store desiccated
   b. *L-Carnitine-d_3* HCl (methyl-d_3), Cambridge Isotope Laboratories Inc. DLM-1871-0.1, store refrigerated
   c. *Choline Chloride*, USP Reference Standard Cat. No. 1133547
   d. *Choline Chloride-d_9* (Trimethyl-d_9 98%) DLM-549-1 Cambridge Isotope Laboratories Inc.

D. Solution Preparation
   a. *Mobile phase.*—
      i. *Mobile phase A* — 10 mM ammonium acetate (95/5 water/acetonitrile v/v). Quantitatively transfer 770 (±15) mg ammonium acetate into a 1 liter glass bottle and dissolve with 950 mL laboratory water. Adjust pH up to 8.2 ~ 8.6 by ammonium hydroxide (B a. iv). Record the amount of ammonium hydroxide added. Add 50 mL acetonitrile to the bottle and mix well. Store room temperature. Expiration: one week.
      ii. *Mobile phase B* — 10 mM ammonium acetate (5/95 water/acetonitrile 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. Transfer the 50 mL solution into a 1 liter glass bottle quantitatively. Add 950 mL acetonitrile into the bottle and mix well. Store room temperature. Expiration: 2 weeks.
   b. Native Stock Solutions
      i. Choline Hydroxide (approximately 3000 µg/mL) Store at 2-8ºC. Expiration: 1 month (currently trying to extend to 1 year).
         1. Dry approximately 200 mg of choline chloride USP reference standard at in a vacuum oven at 65 ºC (±3 ºC) for 4 hours
         2. Weigh 170 mg (± 17 mg) into an appropriate weighing boat.
         3. Transfer into a 50 mL volumetric flask quantitatively.
         4. Dissolve and bring to volume with laboratory water.
      ii. L-Carnitine (approximately 750 µg/mL) Aliquot the stock solution into 2 mL plastic vials with each vial receiving at least 1 mL and store ≤ -15ºC. Expiration: 1 year.
         1. Weigh 150.0 mg ± 15 mg into an appropriate weighing boat as quickly as possible
         2. Transfer into a 200 mL volumetric flask.
         3. Dissolve and bring to volume with laboratory water.
         4. Correct weight for moisture content.
   c. Internal Standard stock Solutions
      i. Choline Chloride-d_9 (approximately 20,000 µg/mL) Aliquot the stock solution into 2 mL plastic vials with each vial receiving about 1 mL and store ≤ -15ºC. Expiration: 1 year.
         1. Transfer 1 gram of d_9-choline chloride into a 50 mL volumetric flask.
         2. Dissolve and bring to volume with laboratory water
      ii. L-Carnitine Chloride-d_3HCl (approximately 10,000 µg/mL) Aliquot the stock solution into 2 mL plastic vials with each vial receiving about 1 mL and store ≤ -15ºC. Expiration: 1 year.

1. Transfer 0.5 grams L-Carnitine -d_3 to a 50 mL flask
2. Dissolve and bring to volume with laboratory water

d. *Mixed Intermediate Internal Standard Solution (MIX-IS).* Prepare fresh on the day of analysis - must use same MIX-IS solution for both calibration working standards and samples.

i. Transfer 400 µL of each -choline-chloride-d_9 and 400 µL-carnitine-d_3 HCl stock solution by a calibrated pipette into a 10 mL volumetric flask
ii. Bring to volume with laboratory water and mix well.

e. *Intermediate Working Standards (IWS)* – Prepare fresh on the day of analysis

i. Pipette 100 µL of the mixed intermediate Internal Standard solution (MIX-IS) into three individual 50 mL volumetric flasks.
ii. Using Table 1, add the required volumes of the stock choline and carnitine solution by proper sizes calibrated pipets.
iii. Bring to volume with laboratory water and mix well.

Table 1. Volumes of each Stock Solution for IWS

	Stock Volume (µL)	MIX-IS (µL)	Final Volume (mL)
IWS -1	25	100	50
IWS -2	125	100	50
IWS -3	250	100	50

f. *Working Standard Solution (WS)* – Prepare fresh on the day of analysis. Use 90% acetonitrile in final working solution.

i. Pipette 200 µL of each Intermediate Working Standards (IWS-1, 2, and 3) into three10 mL volumetric flasks
ii. Add 800 µL laboratory water each volumetric flask
iii. Bring to volume with acetonitrile and mix well
iv. Transfer to autosampler vials for analysis.

Table 2. Concentration of Standards

Name	Choline	Choline-d_9	Carnitine	Carnitine-d_3	Diluent	VF
	µg/mL					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%	
WS1	30	32	7.5	16	MeCN	10
WS2	150	32	37.5	16	MeCN	10
WS3	300	32	75	16	MeCN	10

E. *Procedure* – Select sample weights between 1 and 5 grams based on expected concentration of the two nutrients in each sample. Adjust the final volume to 10 mL (sample + acid +water = 10 mL) prior to microwave digestion which makes acid concentration around 3.5 N.

a. *Powder Products*

i. Powder products are reconstituted with water prior to analysis, typically 11.1% w/w (25g powder added to 200g water)

b. *Sample Analysis*

i. Weigh sample into a tarred microwave reaction vessel
ii. Add 125 µL of the mixed intermediate internal standard solution
iii. Add water to the sample to achieve a total sample volume of 10 mL after the addition of the nitric acid.
iv. Add 2.2 mL 70% nitric acid and seal the vessels
v. Vortex the vessels for 20 seconds, then put caps on.
vi. Complete microwave digestion using the conditions defined in Table 3.

Table 3. Microwave Digestion Parameters

1	Power	100 % (1600 W)
2	Ramp to temperature	10 minutes
3	Hold time	40 minutes
4	Temperature	120 ºC

vii. Uncap each vessels after cool down (by air) , add 3.5 mL concentrated ammonium hydroxide to each vessels.
viii. Vortex each sample for 20 seconds
ix. Allow the samples to stand in a hood for 30 minutes.
x. Vortex the samples for 20 seconds
xi. Transfer 1 mL of the digested sample into a disposable centrifuge tube containing 25 mL laboratory water
xii. Vortex for 20 seconds
xiii. Filter ~ 1 mL of the diluted sample using a 0.45 µm nylon syringe filter
xiv. Transfer 0.1 mL of this filtered solution into an autosampler vial containing 0.9 mL acetonitrile. Mix well. The sample is ready for analysis.
xv. Samples diluted into acetonitrile are stable for 24 hours when stored on a refrigerated (8ºC) autosampler. Filtered samples (aqueous) and WS are stable for 7 days stored 2-8ºC.

F. Instrument Operating Conditions

a. *Mass Spectrometry Conditions* - See Tables 4 and 5
b. *UPLC Conditions* – See Table 6
c. *UPLC analysis*.—Column stability was improved by storing the column in water / acetonitrile 5/95 (v/v) without additives (recommended by column supplier). After verifying equilibration of the UPLC system, inject the mid-level working standard 4 times to verify the system suitability. The %RSD of the peak areas from these injections should be less than 5%. Once system suitability has been established, inject

working standards (WS1 to WS3) followed by a reagent blank, control sample, and samples.

d. Re-inject working standards approximately every 4 hr. (e.g., enough time for 16 samples with analysis cycle time of 15 min). Correlation coefficient of each set of calibration curves should have r^2 >0.9990 and calibration curve residuals should be ≤4%. Samples should be bracketed by two sets of such valid calibration curves.

Table 4. Mass analysis parameters

	Retention time typical, min	Molecular ion (precursor)	Product ion	Dwells	Cone voltage	Collision energy
Choline	2.5	104.2	60.3	0.025	40	12
Choline-d_9	2.5	113.4	69.3	0.025	40	15
Carnitine	4.4	162	103	0.025	32	15
Carnitine-d_3	4.4	165	103	0.025	30	15

Table 5. Mass Spectrometer (Xevo TQ-S) operating conditions

Ionization Mode:	ESI positive
Capillary voltage:	3.6 KV
Collision Gas Pressure	3 to 4 e^{-3}
Source Temperature:	150ºC
Desolvation Temperature:	350ºC
Flow	50 L/hr.
Desolvation Flow Rate	1100 L/hr.
PWHH	0.7 AMU*
Instrumental resolution parameters for the TQ-S are setup by IntelliStart to achieve a resolution of approximately 0.7 amu across the mass range	

Table 6. Chromatographic Parameters

Mobile Phase A	10 mM ammonium acetate (95/5 water/ acetonitrile v/v)
Mobile Phase B	10 mM ammonium acetate (5/95 water/ acetonitrile 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 °C
Injection Volume	10 µL
Injection Type	Full Loop
Sample temperature	8 °C

Table 6. continued

Gradient Profile				
Time (min)	Flow (mL/min)	% MP A	% MP B	Curve
0.1	0.7	8	92	6
6	0.7	21	79	6
6.01	0.7	100	0	6
8	0.7	100	0	6
10	1	8	92	6
13	1	8	92	6
13.2	0.7	8	92	6
15	0.7	8	92	6

NOTES:

1. Free choline or carnitine can be determined by weighing same sample size (as for microwave digest procedure) into a disposable tube, skipping the microwave digestion and filtering the sample at an equivalent dilution (neither ammonia or nitric acid to be added).
2. Unless the sample contains milk proteins, free carnitine = total carnitine (through microwave digestion). Thus, the method is able to determine free and total carnitine and total choline simultaneously through microwave digestion. The non-MW prep is typically used for free choline in products (rare) or to determine free choline/carnitine in premixes. Note that the typical control sample, SRM 1849a, has substantially different values for carnitine depending on whether the SRM is microwave digested.
3. System suitability measures: the residuals must be <4% in each calibration curve; a control sample (SRM 1849a) must be run with each set of samples and results within limits of lab control chart.
4. The authors acknowledge help from Jim Denison, Nick Cellar, and Wes Jacobs in completing this work.

STAKEHOLDER PANEL ON INFANT FORMULA AND ADULT NUTRITIONALS (SPIFAN)

Meeting held at
Hilton Washington DC North/Gaithersburg, MD
Wednesday, March 19, 2014 - 3:00pm (Eastern US)

REPORT OF THE EXPERT REVIEW PANEL (ERP) PROCEEDINGS

Expert Review Panel Members (in attendance):

Darryl Sullivan	***Covance Labs*** *(Chair)*
John Austad	Covance Labs
Sneh Bhandari	Mérieux NutriSciences & OMB
Esther Campos-Gimenez/Adrienne McMahon	Nestlé
Brendon Gill	Fonterra
Don Gilliland/Karen Schimpf	Abbott Nutrition
Estela Kneeteman	INTI
Bill Mindak	FDA
Shay Phillips	Mead Johnson
Guenther Raffler	CLF-Eurofins
Kate Rimmer/Melissa Phillips	NIST
Matt Sliva (for Scott Christiansen)	Perrigo/PBM Nutritionals
Jinchuan Yang	Waters Corp.

Expert Review Panel Members (unable to attend):

Scott Christiansen	Perrigo/PBM Nutritionals
Jon DeVries	General Mills/Medallion Labs
Sarwar Gilani	Consultant
Min Huang	Frontage Labs
Maria Ofitserova	Pickering Labs
Jeanne Rader	FDA (CFSAN)

AOAC Staff Includes:
Delia Boyd
E. James Bradford
Scott Coates
Dawn Frazier
Deborah McKenzie
Alicia Meiklejohn
Tien Milor
Anita Mishra
Bob Rathbone

I. WELCOME AND INTRODUCTIONS

Darryl Sullivan welcomed all participants to the ERP meeting and introduced the ERP members.

II. REVIEW ACTIONS FROM PREVIOUS MEETING

Darryl Sullivan reviewed previous actions/items from past meetings.

III. REVIEW OF METHODS BY EXPERT REVIEW PANEL (ERP) FOR FIRST ACTION *OFFICIAL METHOD*^{SM} STATUS

For each method, the ERP/Working Group Co-Chairs discussed methods submitted.

Carnitine – *Co-Chairs:* John Austad (Covance) & Guenther Raffler (CLF-Eurofins)
Choline – *Co-Chair:* Sneh Bhandari (Silliker) & Nick Cellar (Abbott)

To study directors, methods should be able to detect both carnitine and choline, but evaluate the methods independently.

IV. REVIEW OF MODIFICATION FOR OMA# 2011.08 (Vitamin B_{12})

V.

The Expert Review Panel (ERP) reviewed the modification to OMA method 2011.08. The primary (Christiansen/Sliva) and secondary (Kneeteman) reviewers provided their evaluation along with other members of the ERP.

- This is a new method, it contains significant changes. Should check if it should be major or minor modification
- For AOAC First Action, the method must have SPIFAN SLV data
- For publication, reference the previous method
- Vote the current modified method as AOAC First Action
- HPLC/ULC – have data reflect both HPLC & ULC
- Have a dispute resolution method, but keep as a back-up
- Caveat – back up method if study director doesn't want to continue
- Re-introduce as a new method

VI. REPEAL OF FIRST ACTION METHODS

The Expert Review Panel (ERP) members collectively discussed the next steps on the remaining First Action *Official Methods*^{SM} currently in the SPIFAN system.

Method	Method Title	Reviewer(s)	Vote	Comments
Folate	*2011.05 - Folate in Infant Formula and Adult/Pediatric Nutritional Formula Optical Biosensor Assay*	Sneh Bhandari Melissa Phillips	*Motion to repeal Yes- 11/ No-0 /Abstain-1	◊ Method is being used, but no SLV data to be produced
	2011.06 - Total Folates in Infant Formula and Adult Nutritionals by Trienzyme Extraction and UPLC-MS/MS Quantitation: First Action 2011.06	Adrienne McMahon Matt Sliva for Scott Christiansen	*Motion to extend method for one (1) year Yes- 10/ No-0 /Abstain-1	◊ No further work will be done ◊ Extend method for one(1) year ◊ If method is kept, someone should take it on ◊ 1st Action status should have meaning ○ Proprietary method (single source)
Nucleotides	*2011.21 - Development and Application of an HILIC-MS/MS Method for the Quantitation of Nucleotides in Infant Formula*	Estela Kneeteman Min Huang	*Motion to keep Yes- 11/ No-0 /Abstain-1	◊ Keep as a back-up method

Vitamin A	*2011.07 - Vitamin A in Infant Formula and Adult Nutritionals UPLC-UV*	Don Gilliland Jinchuan Yang	*Motion to keep method Yes- 11/ No-0 /Abstain-1	◊ Keep method ◊ Desire for combo method ◊ Has SLV data, but not published
	2011.15 - Vitamin A (Retinol) in Infant Formula and Adult Nutritionals Liquid Chromatography	Sneh Bhandari Adrienne McMahon	*Motion to repeal Yes- 11/ No-0 /Abstain-1	
Vitamin D	*2011.12 - Vitamins D_2 and D_3 in Infant Formula and Adult Nutritionals Ultra Pressure Liquid Chromatography with Mass Spectrometry Detection (UPLC-MS/MS)*	Brendon Gill Adrienne McMahon	*Motion to repeal Yes- 10/ No-0 /Abstain-1	
	2011.13 - Vitamins D_2 and D_3 in Infant Formula and Adult Nutritionals LC-MS/MS	Matt Sliva for Scott Christiansen Shay Phillips	*Motion to repeal Yes- 9/ No-0 /Abstain-1	◊ Original method was withdrawn and resubmitted
Vitamin B12	*2011.08 - Improved AOAC First Action 2011.08 for the analysis of vitamin B_{12} in Infant Formula and Adult/Pediatric Formulas. Single Laboratory Validation* ***(Modification)***	Bill Mindak Matt Sliva for Scott Christiansen Brendon Gill Shay Phillips	*Motion to give the method a new number (TBD) Yes- 11/ No-0 /Abstain-1 *Motion to repeal old number (2011.08) Yes- 10/ No-0 /Abstain-1	◊ Keep or give it a new number? ◊ Reference back to the old number from the AOAC Journal ◊ ***See section 4 for additional comments***
	2011.09 - Vitamin B_{12} in Infant Formula and Adult Nutritionals HPLC After Purification on an Immunoafinity Column	Matt Sliva for Scott Christiansen Estela Kneeteman	*Motion to keep Yes- 11/ No-0 /Abstain-1	◊ Keep the method
	2011.16 - Vitamin B_{12} in Infant Formula and Adult Nutritionals Surface Plasmon Resonance	Shay Phillips John Austad	*Motion to repeal Yes- 10/ No-0 /Abstain-1	

VII. DISCUSS REQUIREMENTS/EXPECTATIONS RELATED TO MULTI-LABORATORY TESTING REPORTS, AN UPDATE ON MULTI-LABORATORY TESTING AND SCHEDULE

Robert Rankin (International Formula Council) discussed the template to be used for Multi-Laboratory Testing (MLT) reports; the reports will be in a standardized format. The draft template and will be available soon, it's currently in review with the Methods Committee on Statistics.

VIII. UPCOMING METHOD AUTHOR ORIENTATION

Deborah McKenzie, AOAC provided an overview of the upcoming method author orientation including requirements for submission of Multi-Laboratory Testing (MLT) reports.

IX. NEXT STEPS/FEEDBACK FROM ERP

Darryl Sullivan provided next steps including deadline dates. The study directors will need to complete the evaluation sheets and the ERP may have seven (7) MLT reports for review with a mid-July deadline, while providing the ERP with four (4) weeks to complete a thorough review. AOAC will need to be informed if ERP members will be unable to attend the AOAC Annual Meeting in Boca Raton, FL. No feedback/comments were received from the ERP pertaining to the meeting.

AOAC INTERNATIONAL

STAKEHOLDER PANEL ON INFANT FORMULA AND ADULT NUTRITIONALS (SPIFAN)

Meeting held at

Boca Raton Resort & Club

Tuesday, September 9, 2014 - 8:30am (Eastern US)

REPORT OF THE EXPERT REVIEW PANEL (ERP) PROCEEDINGS

Expert Review Panel Members (in attendance):

Darryl Sullivan	***Covance Labs*** *(Chair)*
John Austad	Covance Labs
Sneh Bhandari	Mérieux NutriSciences & OMB
Esther Campos-Gimenez/Adrienne McMahon	Nestlé/Wyeth Nutrition (formerly Pfizer)
Scott Christiansen	Perrigo/PBM Nutritionals
Jon DeVries	General Mills/Medallion Labs
Brendon Gill/Harvey Indyk	Fonterra
Sarwar Gilani	Consultant
Min Huang	Frontage Labs, Inc.
Estela Kneeteman	INTI
Maria Ofitserova	Pickering Labs, Inc.
Melissa Phillips/Kate Rimmer	NIST
Shay Phillips	Mead Johnson Nutrition
Guenther Raffler	CLF-Eurofins
Karen Schimpf	Abbott Nutrition
Scott Christiansen	Perrigo/PBM Nutritionals
Jinchuan Yang	Waters Corp.

Expert Review Panel Members (unable to attend):

Don Gilliland	Abbott Nutrition
William Mindak	FDA
Jeanne Rader	FDA (CFSAN) - Retired

AOAC Staff Includes:

Delia Boyd
E. James Bradford
Scott Coates
Deborah McKenzie
Tien Milor
Anita Mishra
Bob Rathbone

Observers:

Martin Alewijn, *RIKILT*
Sean Austin, *Nestlé Research Center*
Brad Barrett, *ABSciex*
Christopher J. Blake, *Nestlé Research Center*
Martin Bucknall, *UNSW*
Marti Cenky, *Abbott Nutrition*
France Cho, *Maxxam Analytics Inc.*
Mark Collison, *Archer Daniels Midland Co.*
Hans Cruijsen, *FrieslandCampina Domo*
Brian De Borba, *Thermo Fisher Scientific*
Jean-Luc Deborde, *SCL Laboratoire de Strasburg*
Rachel de Guzman, *Mead Johnson Nutrition*
Marieke de Laat, *Mead Johnson Nutrition*
XiaoJun Deng, *SHCIQ*
Marcel deVreeze, *NEW/ISO*
Aurélie Dubois-Lozier, *IDF*
Wayne C. Ellefson, *Covance Laboratories*
Jaap Evers, *ISO Rep. (Fonterra Co-op.)*
Ping Feng, *Consultant*
Jennifer Fruth, *Mead Johnson Nutrition*
Pierre-Alain Golay, *Nestlé Research Center*
Phillip Haselberger, *Abbott Nutrition*
Melissa Holskey, *Abbott Nutrition*
Steve Holroyd, *IDF Rep. (Fonterra Co-op.)*
Gregory Hostetler, *Perrigo/PBM Nutritionals*
Wesley Jacobs, *Abbott Nutrition*
Greg Jaudzems, *Nestlé*
Elaine Jobgen, *Eurofins*
George Joseph, *AsureQuality*
Bert Klarenbeek, *FrieslandCampina Domo*
Khammawan Kohler, *Covance Laboratories*
Erik J. M. Konings, *Nestlé Research Center*
Li Xian Liang, *China ChongQing CIQ*
Stephen Lock, *ABSciex*
E. Marley, *R-Biopharm Rhome Ltd. Scotland*
Frederic Martin, *Nestlé Research Center*
Josh Messerly, *Eurofins*
Deepali Mohindra, *Thermo Fisher Scientific*
Nancy Montgomery, *Abbott Nutrition*
Mardi Mountford, *IFC*
Norriel Nipales, *Wyeth Nutrition (formerly Pfizer)*
Lawrence Pacquette, *Abbott Nutrition*
Shang-Jing Pan, *Abbott Nutrition*
Eric Poitevin, *Nestlé Research Center*
Al Poland, *AOAC Consultant*
Robert Rankin, *IFC*
Lars Reimann, *Eurofins*
Joe Romano, *Waters Corporation*
Steve Royce, *Agilent Technologies, Inc.*
Louis Salvati, *Abbott Nutrition*
Dan Schmitz, *Abbott Nutrition*
Matthew Sliva, *Perrigo/PBM Nutritionals*
Angela Song, *Abbott Nutrition*
Karla Steele, *Mead Johnson Nutrition*
John Szpylka, *Merieux NutriSciences*
Joseph J. Thompson, *Abbott Nutrition*
Linda Thompson, *Abbott Nutrition*
Melissa Thompson, *Covance Laboratories*
Laszlo Torma, *Pickering Laboratories, Inc.*
Marina Torres Rodriguez, *LATU*
Martijn Vermeulen, *TNO*
Wayne Wargo, *Abbott Nutrition*
Guy Weerasekera, *Mead Johnson Nutrition*
Laura Wood, *NIST*
David Woollard, *Hill Laboratories*
Wayne Wolf, *USDA (Retired)*
Jinchuan Yang, *Waters Corporation*
Linda Zhao, *Abbott Nutrition*
Yang Zhou, *Eurofins*

I. WELCOME AND INTRODUCTIONS

Darryl Sullivan welcomed all participants to the ERP meeting and introduced the ERP members.

II. REVIEW OF METHODS BY EXPERT REVIEW PANEL (ERP) FOR FIRST ACTION *OFFICIAL METHOD*^{SM} STATUS

For each method, the ERP/Working Group Co-Chairs discussed methods submitted.

- **Folate – *Chair:*** Erik Konings (Nestlé)

  Fol-20: Method was withdrawn for additional information

- **Carnitine – *Co-Chairs:*** John Austad (Covance) & Günther Raffler (CLF-Eurofins)
  Choline – *Co-Chair:* Sneh Bhandari (Silliker) & Nick Cellar (Abbott)
  - Instructions to study directors to collect choline data as well as carnitine

Method	Method Title	Reviewer(s)	N Vote	Comments
Folate	*Fol-20 - Analysis of Folic acid and 5-Methyltetrahydrofolate in Infant and Adult Nutritional formula using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry*	Adrienne McMahon Min Huang	Method withdrawn from consideration	◊ SPIFAN matrices did not produce internal peaks (repeat work) ◊ Concerned with interferences ◊ Confirmation ion is required ◊ Did not meet LOQ
Carnitine	*Carn-05 - Single Laboratory Validation of CARN-05: Determination of Free and Total Carnitine and Choline in Infant Formulas and Adult Nutritional Products*	John Austad Sneh Bhandari	John Austad moved & Sneh Bhandari second *Motion: move method to First Action *Official Method*^{SM} status **Yes- 14/ No-0 /Abstain-1**	◊ Collect choline data as well as carnitine ◊ Will not be considered dispute resolution method for choline ◊ Investigate choline adult powder milk
	Carn-06 - Simultaneous Determination of Carnitine and choline in Infant Formula, Adult/Pediatric Nutritional Formula, food and feed ESI LC-MS/MS	Günther Raffler John Austad	Method not recommended at this time	◊ Collect choline data as well as carnitine ◊ Recoveries needed at different levels ◊ Must use SPIFAN matrices ◊ Need SPIFAN data ◊ Resubmit for March 2015

III. REVIEW OF METHODS BY EXPERT REVIEW PANEL (ERP) FOR FINAL ACTION *OFFICIAL METHOD*^{SM} STATUS

The Expert Review Panel (ERP) reviewed seven (7) methods for Final Action *Official Methods*^{SM} status. Six (6) were recommended for Final Action to the Official Methods Board (OMB).

Method	Method Title	Reviewer(s)	Vote	Comments
Vitamin B_{12}	*2011.10 - Vitamin B_{12}: - Determination of Vitamin B12 in Infant Formula and Adult Nutritionals by HPLC*	John DeVries Shay Phillips	John DeVries moved & Scott Christiansen second *Motion: move to Final Action Yes- 11/ No-1 /Abstain-3 ——————— *Second vote: Yes- 11/ No-1 /Abstain-3	◊ Question about SPE overload – How to qualify ◊ Include safety for cyanide ◊ Chromatography resolution
Vitamin D	*2011.11 - Vitamin D - Determination of Vitamin D2 and D3 in Infant and Adult/Pediatric Nutritionals and Utilizing Ultra High Performance Liquid Chromatography/Tandem Mass Spectrometry (UHPLC-MS/MS)*	Sneh Bhandari Brendon Gill/ Harvey Indyk	Sneh Bhandari moved & Sarwar Gilani second *Motion: move to Final Action ——————— ~Motion: withdrawn	◊ Editorial changes ◊ Method not a significant improvement over existing method(s) ◊ 50% of samples do not apply to SMPR ◊ Qualifier removed ◊ Single platform – Blinded
Inositol	*2011.18 - Inositol - Determination of Myo-Inositol (Free and Bound as Phosphatidylinositol) in Infant Formula and Adult Nutritionals by Liquid Chromatography/Pulsed Amperometry with Column Switching*	Brendon Gill/ Harvey Indyk	Esther Campos-Gimenez moved & Melissa Phillips second *Motion: move to Final Action Yes- 12/ No-1 /Abstain-2 ——————— *Second vote: Yes- 12/ No-1 /Abstain-2	◊ Remove phosphatidylinositol (data does not support) ◊ Method does not match SMPR ◊ Sum of free myo-inositol (change total to free in method) ◊ Remove "phosphorylated forms" from applicability section ◊ Sum and Free and phosphydal Inositol; c**apture in applicability statement of SMPR.**
UTM	*2011.19 - Ultra Trace Minerals - Simultaneous Determination of Chromium, Selenium, and Molybdenum in Infant Formula and Adult/Pediatric Nutritional Formula by Inductively Coupled Plasma/Mass Spectrometry*	Min Huang Sneh Bhandari	Min Huang moved & Sneh Bhandari second *Motion: move to Final Action Yes- 13/ No-0 /Abstain-1	
Nucleotides	*2011.20 - Nucleotides - Nucleotides in Infant Formula by HPLC-UV*	Sneh Bhandari Estela Kneeteman	Sneh Bhandari moved & Estela Kneeteman second *Motion: move to Final Action Yes- 13/ No-0 /Abstain-1	

Vitamin A/E	*2012.10 - Vitamin A/E - 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 DL-α-Tocopherol Acetate) in Infant Formula and Adult Nutritionals by Normal Phase HPLC*	Scott Christiansen Jinchuan Yang	Scott Christiansen moved & Brendon Gill second *Motion: move to Final Action Yes- 13/ No-0 /Abstain-1	◊ Require use of primary standards ◊ Simplify calibration curve (all trans)
Fatty Acids	*2012.13 - Fatty Acids - Determination of Fatty Acids, including LCPUFAs, in Infant and Adult/Pediatric Nutritional Formula*	Jon DeVries Karen Schimpf	Jon DeVries moved & Karen Schimpf second *Motion: move to Final Action Yes- 13/ No-0 /Abstain-1	◊ Reference materials with higher RSD ◊ Double check chromatography for clarity (peaks)

IV. NEXT STEPS/FEEDBACK FROM ERP

Darryl Sullivan provided next steps including deadline dates. The ERP provided feedback on lessons learned from reviews.

AOAC Official Method 2011.10
Vitamin B_{12} in Infant and Pediatric Formulas and Adult Nutritionals
High-Performance Liquid Chromatography
First Action 2011
Final Action 2014

(Applicable to the determination of vitamin B_{12} in infant and pediatric formulas and adult nutritionals.)

Caution: Refer to Material Safety Data Sheets (MSDS) of chemicals prior to use and follow the suggested personal protective equipment.

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 local, state, and federal regulations.

See Tables **2011.10A–C** for results of the single-laboratory validation and multilaboratory 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} solid-phase extraction (SPE) cartridges and analyzed with size-exclusion and reversed-phase chromatography. Determination of B_{12} is made by liquid chromatography with visible detection 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 µ, 250 × 9.4 mm (Zorbax GF-250, P/N 884973-901; www.chem.agilent.com), 5 µ, 300 × 8 mm (Shodex Protein KW-802.5, P/N F6989000) or equivalent.

(**c**) *Column.*—Analytical C_{18} column 3 µ, 100 × 4.6 mm (Thermo Scientific Aquasi1 P/N 77503-104630; www.thermoscientific.com) with C_{18} drop-in guard cartridges 3 µ, 10 × 4.6 mm (Thermo Scientific Aquasil P/N 77503-014001), Epic Phenyl Hexyl, 3 µ, 120A, 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 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 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 P/N 20966), C_{18} 900 mg (Alltech/Grace Davison P/N 20942), or equivalent. *See* **E**, procedure 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.

Table 2011.10A. Single-laboratory validation: 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]	1.37	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 RTF 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.

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Table 2011.10B. Single-laboratory validation: Accuracy (spike recovery) data for vitamin B_{12}

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 placebo	4	0.109	98.2	7.98
Adult nutritional RTF high fat	4	1.59	102	1.61
Infant elemental powder placebo	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.

(**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.

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.

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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.

(*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*) *2.5% Acetonitrile in H_2O (mobile phase D).*—50 mL acetonitrile diluted to 2000 mL with laboratory water. Expiration 1 week in tightly stoppered container.

(*5*) *10% Acetonitrile in H_2O.*—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 H_2O, 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 weigh 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 4.5 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 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 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

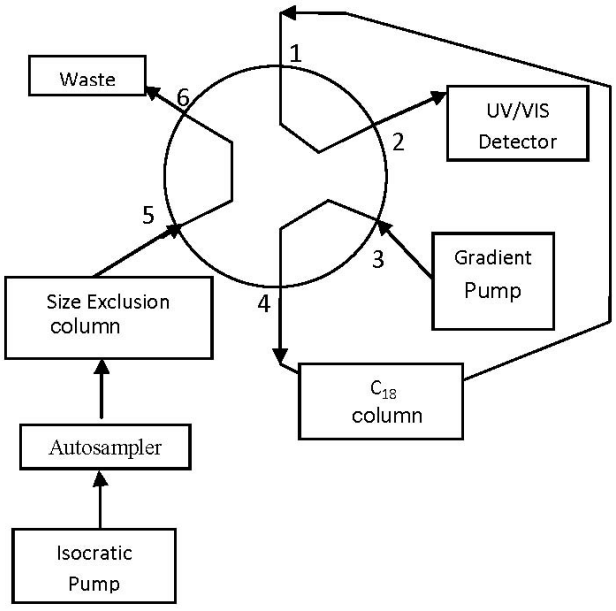

Figure 2011.10A. System setup and configuration: Configuration 1.

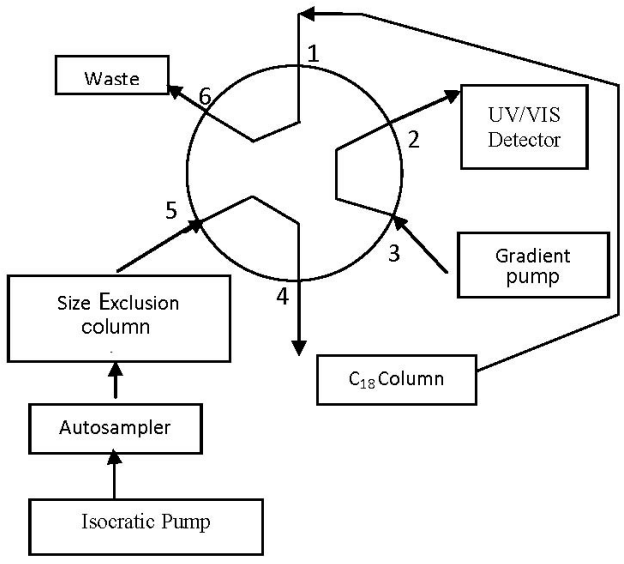

Figure 2011.10B. System setup and configuration: Configuration 2.

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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

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*)–(*2*) of the sample preparation procedure described below.

(*3*) Combine all extracted sample filtrates. Add 1 mL of the solution prepared in step (*1*) to 80 or 100 mL of sample filtrate (spiked sample), and add 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 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 4 significant figures. Typical weights are 20 g for adult and pediatric ready-to-feed (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 of a 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 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 that 0.5 g of milk protein have been added to, 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 gravity filter 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 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 eluant. After all of the sample filtrate has passed through the cartridge, rinse each cartridge with 5 mL laboratory water and discard eluant. 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.

Table 2011.10F. Reversed-phase column 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
27.0–30.0	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. Inject the resolution test solution and calculate the resolution (R) between vitamin B_{12} and riboflavin. Adjust the mobile phase composition between 14.6 and 27.0–30.0 min until R 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.

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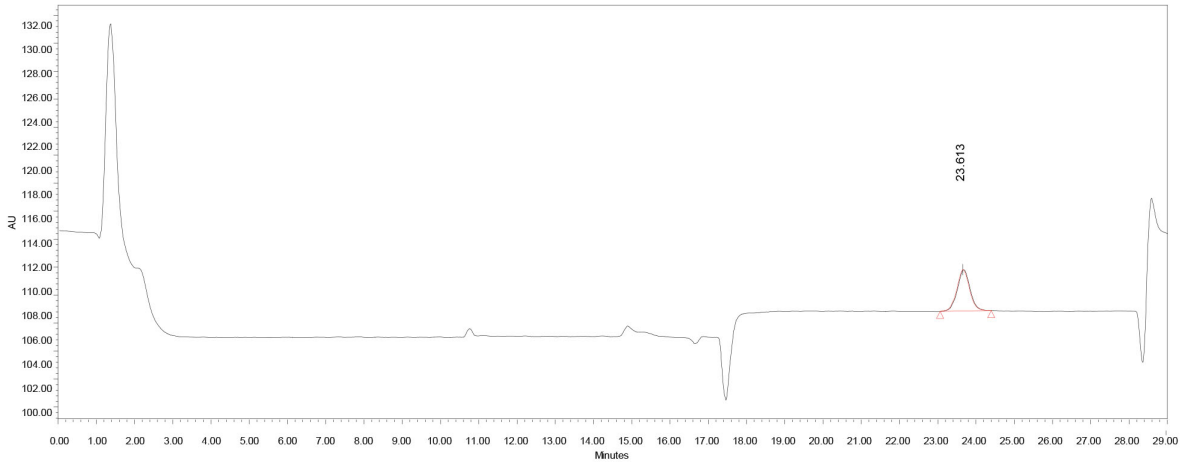

Figure 2011.10C. Typical standard chromatogram.

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.

(**b**) *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**.

(*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, followed by a control sample, 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 **2011.10C** and **D**).

(**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

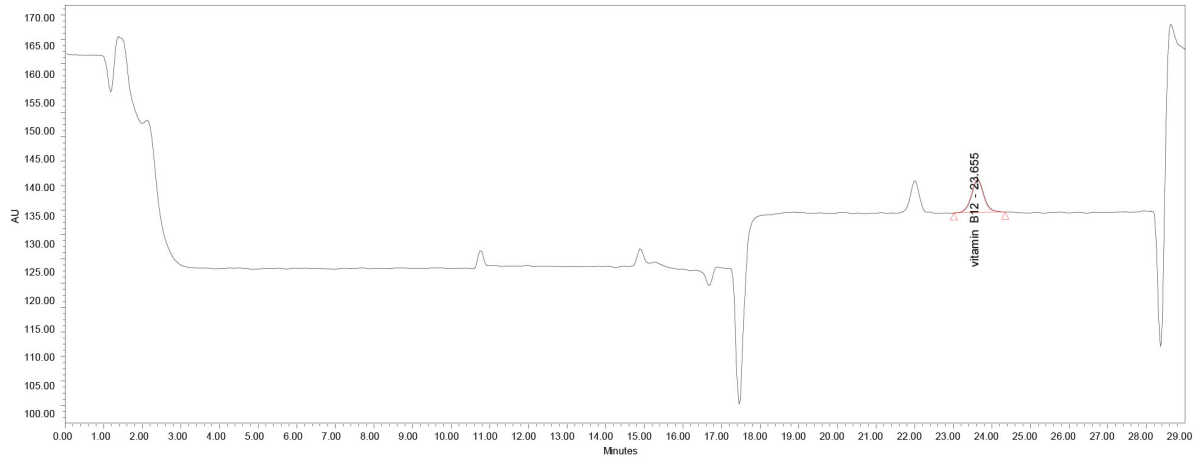

Figure 2011.10D. Typical standard chromatogram.

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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 concentrations*.

$$WS = S_w \times P \times A/200$$

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*.—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) on concentration versus the average peak area of the working standards. A standard curve must have a correlation of at least 0.999 to be considered acceptable for sample calculations.

(**e**) 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%.

(**f**) *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 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; V = volume of filtrate loaded onto the cartridge in mL.

For each set of samples, the control result must be within three standard deviations of the control mean.

References: *J. AOAC Int*. **95**, 313(2012)
DOI: 10.5740/jaoacint.CS2011_10

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J. AOAC Int. **95**, 293(2012)
DOI: 10.5740/jaoac.int.11-0441

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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

The liquid chromatography 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 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 limits of detection and quantitation were determined statistically from injections of low-level standards and by spiking samples with low levels of free myo-inositol.

See Tables **2011.18A–C** for method performance information 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*.

Table 2011.18A. Single-laboratory validation: Repeatability precision data for myo-inositol

Sample type	No. of replicates (duplicates on multiple days)	Mean (mg/100 g RTF)	SD_r	RSD_r
Unbound (free) myo-inositol				
Infant formula (NIST SRM 1849a)	14	415[a]	8.30	2.00
Infant formula powder soy-based	14	4.19	0.091	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
Unbound (free) myo-inositol plus myo-inositol bound as phosphatidylinositol				
Infant formula (NIST SRM 1849a)	12	426a	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.

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Table 2011.18B. Single-laboratory validation: 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
Unbound (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.

(**d**) *N-evap.*—With water bath 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**) *System HPLC*.—Corrosion-resistant components including an autosampler, isocratic pumps (2), 6-port switching valve, pulsed amperometry detector with a gold electrode and PEEK or Teflon 0.007–0.01 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 2V 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) and mechanical; assorted sizes.
(**i**) *Solid-phase extraction (SPE) cartridges.*—Silica, 1 g (J.T. Baker 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.

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 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 of the 0.5 mg/L myo-inositol working standard to 10 mL with laboratory water (0.2 and 0.05 mg/L). Expiration: 2 weeks.

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Table 2011.18C. Interlaboratory study results for myo-inositol

Sample type	Total No. labs	Total No. replicates	Mean (mg/100 g RTF)	SD_r	SD_R	RSD_r	RSD_R	HorRat[a]
Unbound (free) myo-inositol								
Infant formula (NIST SRM 1849a)	10	22	412[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	8	16	4.23	0.0660	0.109	1.56	2.57	0.28
Infant formula RTF milk-based	8	18	7.20	0.0455	0.185	0.63	2.57	0.31
Infant formula powder partial hydrolyzed milk-based	8	18	3.83	0.0368	0.0892	0.96	2.33	0.25
Infant formula powder partial hydrolyzed soy-based	10	22	3.11	0.0899	0.389	2.92	12.61	1.32
Child formula powder	8	18	5.05	0.0233	0.109	0.46	2.15	0.24
Infant elemental powder	9	20	5.13	0.0950	0.277	1.85	5.41	0.61
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)	8	18	9.26[b]	1.08	2.37	11.3	25.0	2.19
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
Unbound (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	8	16	4.94	0.0696	0.302	1.41	6.12	0.69
Infant formula RTF milk-based	7	16	7.53	0.0588	0.201	0.78	2.66	0.32
Infant formula powder partial hydrolyzed milk-based	7	16	4.04	0.0380	0.124	0.94	3.07	0.33
Infant formula powder partial hydrolyzed soy-based	9	20	4.71	0.152	0.357	3.22	7.55	0.84
Child formula powder	8	18	5.42	0.0280	0.285	0.51	5.23	0.60
Infant elemental powder	8	18	5.11	0.0990	0.283	1.94	5.56	0.63
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.

(**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 N.*—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 of 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.

(**g**) *Sodium hydroxide, 4.0% or 1 M (Pump 2).*—Quickly weigh 160 (±3) g of 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.

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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**)

(*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%) of 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 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

Table 2011.18E. PAD settings with gold electrode

Analog range	1 uC	
Detector program: Dionex ICS 3000 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	

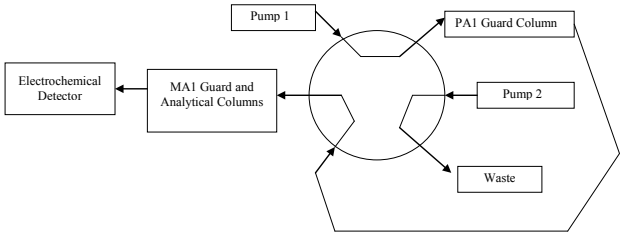

Figure 2011.18A. Switching valve configuration 1.

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 1 mL 1 N 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 eluant. 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 50 mL centrifuge tube. Rinse 50 mL centrifuge tube with 4 mL methanol:chloroform:water (75:15:10) and transfer

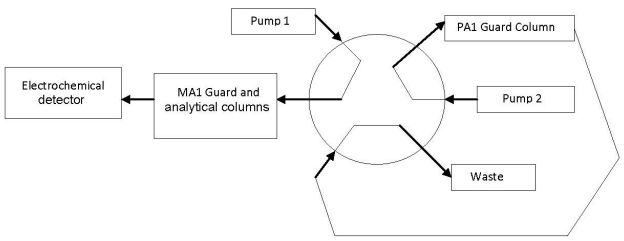

Figure 2011.18B. Switching valve configuration 2.

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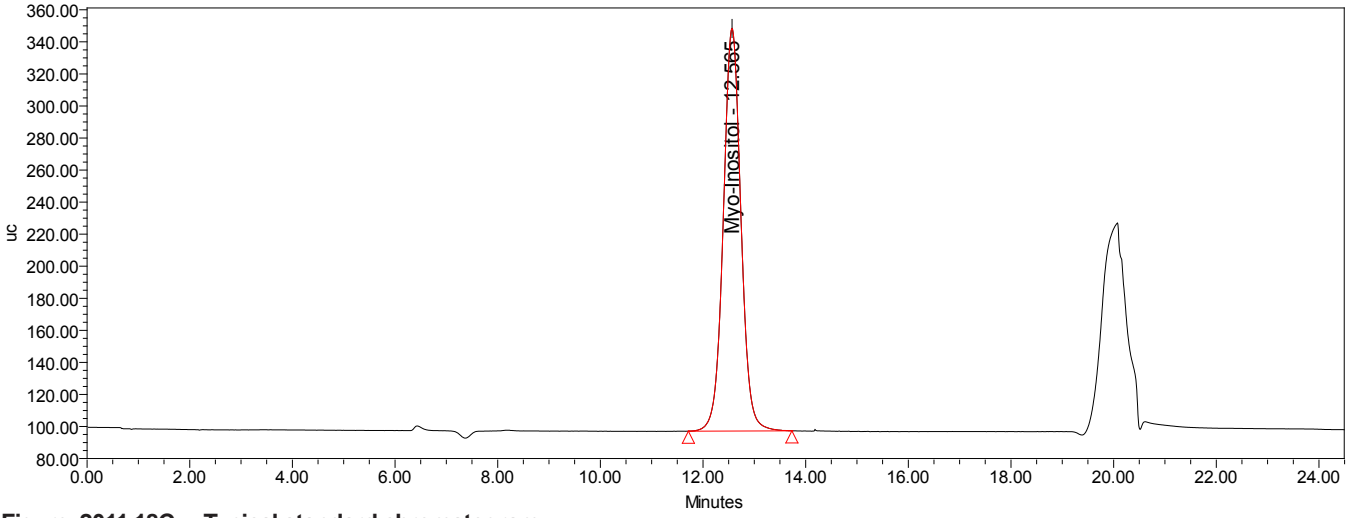

Figure 2011.18C. Typical standard chromatogram.

to the SPE cartridge. Collect eluent in the same 50 mL centrifuge tube. Evaporate eluants 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 residue in 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 **E** 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 over the columns at a flow rate of 0.40 mL/min for at least 1/2 h 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 one vial 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.

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 not any interfering

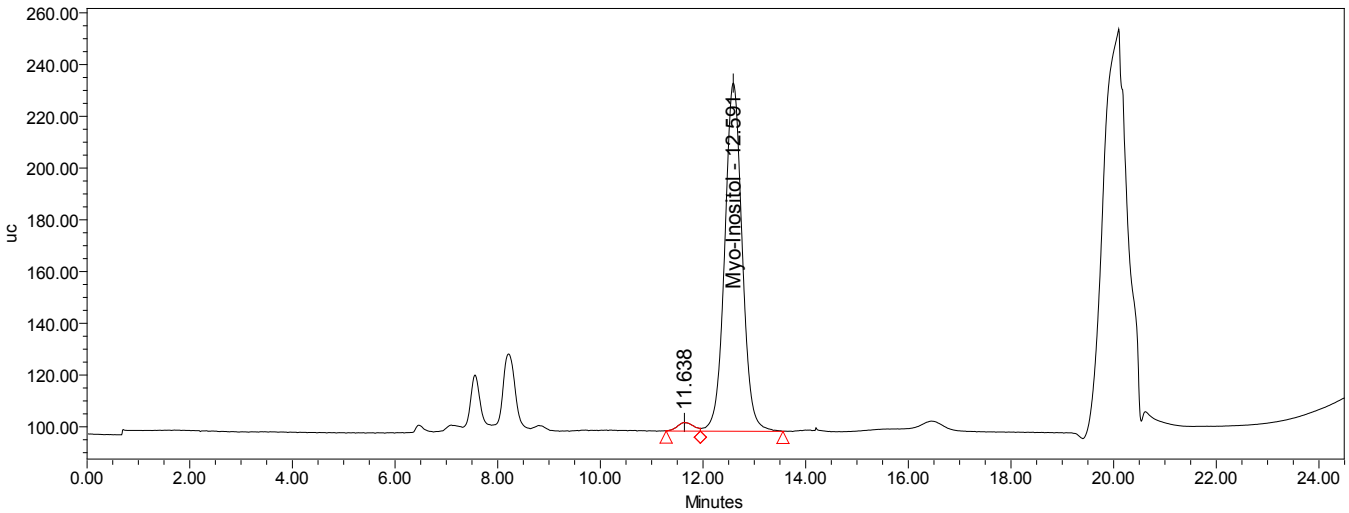

Figure 2011.18D. Typical sample chromatogram.

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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.

(**a**) *Concentration of working standards*.

$$C_W = W \times 1/0.05 \times 1/10 \times A_1/V_1 \times A_2/V_2$$
$$= W \times 2 \times A_1/V_1 \times A_2/V_2$$

where C_W is the concentration of the working standard solution in milligrams per liter; W is the weight, in milligrams, of myo-inositol standard weighed; 0.05 is the dilution volume of the stock standard in liters; 1/10 is the intermediate standard dilution (10 to 100 mL); A_1 is the aliquot of intermediate standard used, in milliliters; V_1 is the dilution volume of the working standard in milliliters; A_2 is the aliquot of working standard used, in milliliters, if applicable; and V_2 is the dilution volume of the working standard in milliliters, if applicable.

(**b**) *Preparation of standard curve.*—For each working standard concentration, average the peak areas or heights from each 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 milligrams per kilogram; C_d is the concentration of myo-inositol in the prepared sample in milligrams per liter; D_1 is the dilution volume in milliliters; and S is the sample weight in grams.

Note: For each set of samples, the control result must be within three standard deviations of the control mean.

See Figures **2011.18C** and **D** for typical standard and sample chromatograms, respectively.

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AOAC SMPR 2011.007
J. AOAC Int. **95**, 295(2012)
DOI: 10.5740/jaoac.int.11-0443

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50.1.41

AOAC Official Method 2011.19
Chromium, Selenium, and Molybdenum in Infant Formula and Adult Nutritional Products

Inductively Coupled Plasma-Mass Spectrometry
First Action 2011
Final Action 2014

A. Principle

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 presented to the inductively coupled plasma-mass spectrometer (ICP-MS) instrument standardized with acid matched standard calibrant solutions. An ionization buffer (potassium) is used to minimize easily ionizable element (EIE) effects, methanol is added to normalize the carbon content, and germanium and tellurium are used as internal standards. 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, Mn.

B. Apparatus

(**a**) *Microwave.*—Commercial microwave designed for laboratory use at 0–300°C, with closed vessel system and controlled temperature ramping capability. It is recommended that the vessel design be selected that will withstand the maximum possible pressure, since organic material, and also 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 (CRCs). In the multilaboratory testing study, five different model ICP-MS instruments from three major vendors delivered equivalent performance.

(**c**) *Various plasticware and pipets.*

C. Chemicals and 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 throughout.

(**c**) *Hydrogen peroxide.*—30% ACS reagent grade.

(**d**) *Methanol.*—99.99% analytical reagent grade for matrix matching.

(**e**) *Potassium.*—10 000 mg/L in nitric acid for matrix matching (may be replaced by multielement standards that contain K, if major minerals are determined simultaneously).

D. Standards

(**a**) *2 mg/L Cr and Mo and 1 mg/L Se multielement stock standard solution in nitric acid.*—High-Purity Standards (Charleston, SC), or equivalent.

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/internal standard/ gas mode	^{52}Cr, $^{95}Mo/^{72}Ge$ in He mode; $^{78}Se/^{130}Te$ in H_2 mode

(**b**) *5 mg/L Ge and Te multielement stock standard solution in nitric acid.*—High-Purity Standards, or equivalent.

(**c**) *SRM 1849a (NIST) or other suitable standard reference material.*—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 internal standards, in HNO_3. Ge is used as the internal standard 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 1-step digestion (two stages in microwave program), add 0.5 mL 5000 ng/mL Ge and Te internal standard solution (with a micropipet calibrated at point-of-use to deliver with at least 0.8% accuracy; do not add the internal standards on-line) 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 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 0.5 mL 5000 ng/mL Ge and Te internal standard solution (with calibrated micropipet as above) and 5 mL trace metal-grade HNO_3. Do not add the internal standards on-line. With power settings appropriate to 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

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Table 2011.19B. Operating parameters

	Stage 1 sample digestion	
1	Power	100% (1600 W)
2	Ramp to temperature	20 min
3	Hold time	20 min
4	Temperature	180°C
5	Cool down	20 min
	Stage 2 sample digestion	
1	Power	100% (1600 W)
2	Ramp to temperature	20 min
3	Hold time	20 min
4	Temperature	200°C
5	Cool down	20 min

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 on-line 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 internal standard for both Cr and Mo (helium mode), and Te must be used for Se (hydrogen mode). Analyze a 4 ng/mL Cr and Mo, and 2 ng/mL Se working standard or other suitable quality control 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 <1/2 of the lowest calibration standard), a duplicate sample [relative percent 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:

$$x = \frac{y - \text{blank}}{a} * \text{DF}$$

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 internal standard 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 internal standard at the same level as the blank standard solution; a = slope of the calibration curve; and DF = dilution factor of the sample solution divided by sample weight (mL/g).

H. Method Validation

(**a**) *Linearity*.—All calibration curves were prepared using a nonweighted least-squares linear regression analysis, 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 linear 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 carbon (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 carbon) 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 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 National Institute of Standards and Technology (NIST) standard reference materials (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-AES and atomic fluorescence spectrometry (AFS) 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- 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

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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 internal standards.

(**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 models of ICP-MS 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 of 0.35 across all three analytes and samples.

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AOAC SMPR 2011.009
J. AOAC Int. **95**, 297(2012)
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AOAC Official Method 2011.20
Nucleotides in Infant Formula

HPLC-UV
First Action 2011
Final Action 2014

(Applicable to the determination of nucleotide 5′-monophosphates in infant 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

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 solid-phase extraction (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 (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).

(**c**) *Spectrophotometer*.—Capable of digital readout to 3 decimal places.

(**d**) *pH meter*.

(**e**) *Centrifuge*.

(**f**) *Ultracentrifuge tubes*.—Amicon MWCO 3k, 4 mL (Millipore-Carrigtwohill, Co. Cork, Ireland).

(**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).

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

C. Reagents

(**a**) *Standards*.—Should be ≥99% pure (Sigma 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)*.

(**g**) *Sodium chloride (NaCl)*.

Table 2011.20A. UV absorbance maxima and extinction coefficients for nucleotide 5′-monophosphates

Nucleotide 5′-monophosphate	λ_{max}, nm	$E^{1\%}_{1cm}$
Adenosine 5′-monophosphate	257	428.6
Cytidine 5′-monophosphate	280	390.9
Guanosine 5′-monophosphate	254	392.0
Inosine 5′-monophosphate	249	356.5
Uridine 5′-monophosphate	262	312.7
Thymidine 5′-monophosphate	267	288.5

(**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 orthophosphoric acid. Dilute to 1 L.

(**b**) *Extraction solution (NaCl 1 M, EDTA 5 mM)*.—Dissolve 58.5 g NaCl and 1.5 g EDTA. Dilute 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 orthophosphoric acid. 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 (about 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.

(**c**) *Internal standard solution (about 80 µg/mL)*.—Dilute 4 mL TMP stock standard into 50 mL water.

Table 2011.20B. Nominal concentration of calibration standards

Calibration solution	Concn AMP, CMP, GMP, IMP, UMP, µg/mL	Concn TMP, µg/mL
1	0.4	3.2
2	0.8	3.2
3	3.2	3.2
4	8.0	3.2

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(**d**) *Working standard solution (about 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 5 mM).

(**d**) Add 1.0 mL TMP internal standard (about 80 µg/mL).

(**e**) Cap the tube and vortex mix until powder dissolves.

(**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 of prepared sample to two separate ultracentrifuge tubes and centrifuge at 3500 × *g* for 60 min, then pool filtrate 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 with 4 mL methanol and draining to top of the cartridge bed; followed by adding two lots of water (5 mL each) and draining to top of 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.

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

(**e**) 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.

(**f**) Filter an aliquot (about 2 mL) of the 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 by the photodiode array detector with chromatograms monitored at the specified wavelengths below for quantitation.

(*1*) IMP at 250 nm.

(*2*) AMP, GMP, and TMP at 260 nm.

(*3*) CMP and UMP at 270 nm.

(**c**) Set column oven to 40°C.

I. Calculations

(**a**) *Concentration of nucleotide in stock standards (SS).*—

$$SS,\ \mu g/mL = \frac{wtSS}{50} \times \frac{PS\%}{100} \times 10^3$$

where *wtSS* = weight of nucleotide in stock standard (mg); 50 = total volume of stock standard (mL); 10^3 = concentration conversion (mg/mL to µg/mL); *PS*% = percent purity; and 100 = mass conversion (% to decimal).

(**b**) *Percent purity of each nucleotide (as free acid) in purity standard (PS).*—

$$\text{Purity, \%} = \frac{\text{Abs}_{\lambda max}}{E^{1\%}_{1cm}} \times \frac{50}{wtSS} \times \frac{50}{1} \times 1000$$

where $Abs_{\lambda max}$ = 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 internal standard (IS).*—

$$IS,\ \mu g/mL = SS \times (4/50)$$

where *SS* = concentration of TMP in stock standard (µg/mL); 4 = volume of TMP stock standard in internal standard (mL); and 50 = total volume of internal standard (mL).

(**d**) *Concentration of nucleotides in working standard (WS).*—

$$WS,\ \mu g/mL = SS \times (2/50)$$

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).*—

$$CS,\ \mu g/mL = IS \times \frac{1}{25}$$

where *IS* = concentration of nucleotide in internal standard (µg/mL); 1 = volume of internal standard in calibration standard (mL); and 25 = total volume of calibration standard (mL).

(**f**) *Concentration of nucleotides in CS.*—

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$$CS, \mu g/mL = WS \times (V_{WS}/25)$$

where WS = concentration of nucleotide in working standard (µg/mL); V_{WS} = volume of working standard in calibration standard (mL); and 25 = total volume of calibration standard (mL).

(**g**) Determine the linear regression curve for the ratio of peaks areas (nucleotide/TMP; y-axis) vs the ratio of concentrations (nucleotide/TMP; x-axis) for calibration standards 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 internal standard added to sample (µg/mL); V_{IS} = volume of internal standard 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 hg or mL to dL).

J. Data Handling

Report results in mg/hg or mg/dL to 1 decimal place.

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J. AOAC Int. **95**, 599(2012)
DOI: 10.5740/jaoacint.CS2011_20

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AOAC Official 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 (dl-α-Tocopherol and dl-α-Tocopherol Acetate) in Infant Formula and Adult Nutritionals

Normal-Phase HPLC
First Action 2012

[Applicable to the concurrent quantitative analysis of total vitamin E (dl-α-tocopherol and dl-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 No. 68-26-8), retinyl esters (retinyl palmitate; CAS No. 79-81-2), and retinyl acetate (CAS No, 127-47-9). The determination of vitamin E focuses on d-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.]

The analytical range of the method is as follows:

Vitamin A, retinyl palmitate.—2–450 µg/100 g reconstituted.

Vitamin A acetate.—2–450 µg/100 g reconstituted.

dl-α-Tocopherol acetate.—0.02–9.4 mg/100 g reconstituted.

dl-α-Tocopherol.—0.03–8.0 mg/100 g reconstituted.

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 (MSDSs) 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.

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 dl-α-tocopherol and dl-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. In order to account for the different biopotency values of the isomers, if required, all analytes are quantified in IUs and converted to µg or mg/100 g reconstituted final product following summation of the isomers.

B. Apparatus

(**a**) *HPLC system*.—Contains pump, autosampler, and programmable UV and fluorescence detectors (FLD), controlled by applicable software, Agilent (Santa Clara, CA) 1200, or equivalent. A Zorbax NH_2, 150 × 4.6 mm id, 5 µm particle size column (Agilent), or equivalent with equal performance was used.

(**b**) *Water bath*.—Capable of 37 ± 2°C.

(**c**) *Centrifuge*.—With adapters for 50 mL centrifuge tubes capable of 4000 rpm.

(**d**) *Laboratory mechanical test tube shaker (optional)*.

(**e**) *UV-Vis spectrophotometer*.—With 1 cm quartz cells.

(**f**) *Standard laboratory glassware*.

(**g**) *Vials*.— 2 mL amber fitted with PTFE liners (Agilent).

(**h**) *Duran bottles*.—1 and 2 L, for the mobile phase (Wertheim/Main, Germany).

(**i**) *Disposable centrifuge tubes*.—50 mL Falcon tubes, or equivalent (Fisher, Pittsburgh, PA).

(**j**) *Disposable Pasteur pipets.*

C. Standards

(**a**) *Vitamin A palmitate.*—Reference standard, Sigma (St. Louis, MO) Cat. No. R3375, or equivalent.

(**b**) *Vitamin A acetate.*—Reference standard, Sigma Cat. No. 46958, or equivalent.

(**c**) *dl-α-Tocopherol acetate.*—Reference standard, Sigma Cat. No. T3376, or equivalent.

(**d**) *dl-α-Tocopherol.*—Reference standard, Sigma Cat. No. 95240, or equivalent.

D. Chemicals and Reagents

(**a**) *Deionized water*.—>18 MΩ resistance (EMD Millipore, Billerica, MA, or equivalent).

(**b**) *Methyl-t-butyl ether*.—HPLC grade (also known as *tert*-butyl methyl ether).

(**c**) *Hexane, ethanol, methanol, and iso-octane (2,2,4-trimethylpentane)*.—HPLC grade.

(**d**) *Papain (from Carica Papain), 3 units/mg*.—Sigma Cat. No. 76220, or equivalent.

(**e**) *Hydroquinone*.—Sigma H9003, or equivalent.

(**f**) *Anhydrous sodium acetate*.—BDH (Visela, CA) 10236, or equivalent.

E. Solutions

(**a**) *2% Papain solution*.—Dissolve 100 mg hydroquinone and 4 g sodium acetate in approximately 80 mL water in a 100 mL volumetric flask. Adjust the pH to 5.0 with dilute hydrochloric acid. Add 2 g papain and make up to volume. Prepare fresh on day of use.

(**b**) *Glacial acetic acid*.—Reagent grade.

(**c**) *Dilute hydrochloric acid*.—100 mL of 37% HCl diluted to 200 mL with distilled water.

(**d**) *Acidified methanol*.—Add 20 mL acetic acid to 1 L methanol and mix. Prepare fresh on day of use.

(**e**) *Mobile phase A*.—Hexane, filtered, and deaerated for 10 min in an ultrasonic bath.

(**f**) *Mobile phase B*.—Hexane–methyl-*t*-butyl ether (75 + 25, v/v). Add 3 mL methanol, filter, and deaerate for 10 min in an ultrasonic bath.

F. Calibration Standards

Note: Class A certified glassware is recommended for the preparation of stock reference standards.

(**a**) *Vitamin A palmitate stock standard (P1)*.—Weigh (to 0.01 mg) approximately 70 mg retinyl palmitate into a 50 mL volumetric flask. Dissolve in and dilute to volume with iso-octane.

(**b**) *Vitamin A acetate stock standard (A1)*.—Weigh (to 0.01 mg) approximately 35 mg retinol acetate into a 50 mL volumetric flask. Dissolve in and dilute to volume with ethanol.

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Table 2012.10. 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

(**c**) *dl-α-Tocopherol acetate stock standard (E1).*—Weigh (to 0.01 mg) approximately 180 mg dl-α-tocopherol acetate into a 50 mL Class A certified volumetric flask. Dissolve in and dilute to volume with iso-octane.

(**d**) *dl-α-Tocopherol stock solution (E2).*—Weigh (to 0.01 mg) 100 mg α-tocopherol into a 50 mL Class A certified volumetric flask. Dissolve in and dilute to volume with iso-octane.

(**e**) *Combined working standard (S3).*—Transfer by pipet 4 mL Pl, 4 mL Al, 7 mL El, and 20 mL E2 into a 50 mL volumetric flask. Dilute to volume with iso-octane, S2. Transfer by pipet 4 mL S2 into a 50 mL volumetric flask and dilute to volume with iso-octane. Store in a refrigerator for up to 7 days.

(**f**) *Level 1 calibration standard.*—Prepare as follows: Into a 50 mL volumetric flask, transfer by pipet 0.5 mL S3, and dilute to volume with iso-octane. Transfer 2 mL into a 10 mL volumetric flask and dilute to volume with iso-octane.

Level 2 calibration standard.—Prepare as follows: Into a 50 mL volumetric flask, transfer by pipet 8 mL S3, and dilute to volume with iso-octane.

Level 3 calibration standard.—Prepare as follows: Use S3 solution.

The standard curve calibration standards for levels 1 and 2 should be prepared daily.

G. Concentration of Stock Standards

(**a**) *Vitamin A palmitate.*—(*1*) Pipet 3 mL stock solution P1 into a l00 mL volumetric flask and make up to volume with iso-octane. Pipet 3 mL this solution into a l00 mL volumetric flask and dilute to volume with iso-octane.

(*2*) Determine the absorption at 325 nm, zeroed against iso-octane in a 1 cm quartz cell.

(*3*) Repeat the reading twice, rinsing the sample cuvet with the solution before each reading. Calculate the average absorbance reading. The potency of the vitamin A palmitate stock solution is then calculated as follows:

$$IU/mL = \frac{Abs}{975} \times \frac{100}{3} \times \frac{100}{3} \times 10000 \times 1.817$$

where *Abs* = average absorbance reading, determined above; 975 = extinction coefficient of retinyl palmitate at 325 nm; 10 000 = conversion of percent to µg/mL; and 1.817 = conversion from µg to IU for retinyl palmitate.

(**b**) *Vitamin A acetate.*—(*1*) Pipet 3 mL stock solution A1 into a l00 mL volumetric flask and make up to volume with iso-octane. Pipet 3 mL this solution into a l00 mL volumetric flask and dilute to volume with iso-octane.

(*2*) Determine the absorption at 325 nm, zeroed against iso-octane, in a 1 cm quartz cell.

(*3*) Repeat the reading twice, rinsing the sample cuvet with the solution before each reading. Calculate the average absorbance reading. The potency of the vitamin A acetate stock solution is then calculated as follows:

$$IU/mL = \frac{Abs}{1560} \times \frac{100}{3} \times \frac{100}{3} \times 10000 \times 2.904$$

where *Abs* = average absorbance reading, determined above; 1560 = extinction coefficient of retinyl acetate at 325 nm; 10 000 = conversion of percent to µg/mL; and 2.904 = conversion from µg to IU for retinyl acetate.

(**c**) *dl-α-Tocopherol acetate.*—(*1*) Pipet 3 mL stock solution E1 into a l00 mL volumetric flask and make up to volume with iso-octane.

(*2*) Determine the absorption at 284 nm, zeroed against iso-octane, in a 1 cm quartz cell.

(*3*) Repeat the reading twice, rinsing the sample cuvet with the solution before each reading. Calculate the average absorbance reading. The potency of the dl-α-tocopherol acetate stock solution is then calculated as follows:

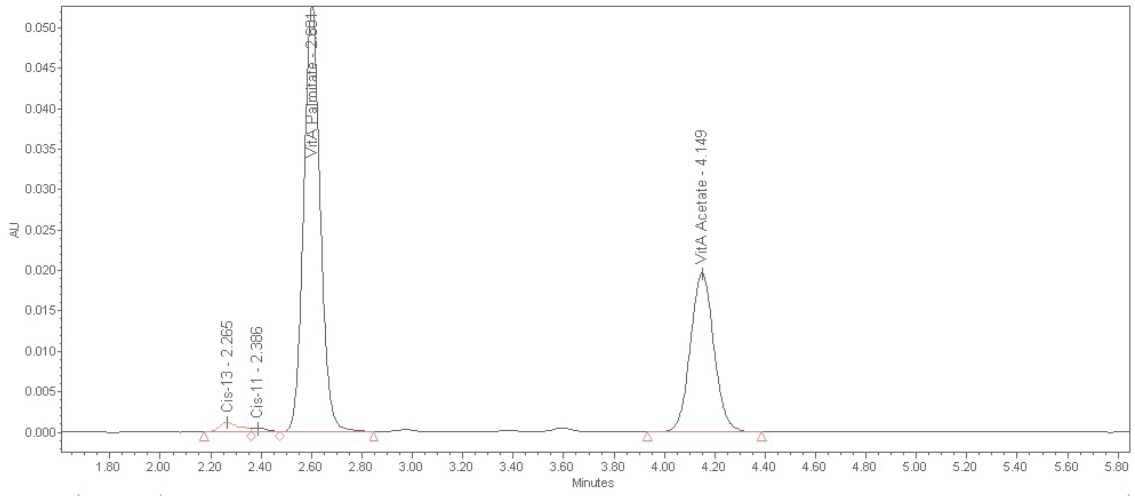

Figure 2012.10A. Vitamin A palmitate and vitamin A acetate calibration standard.

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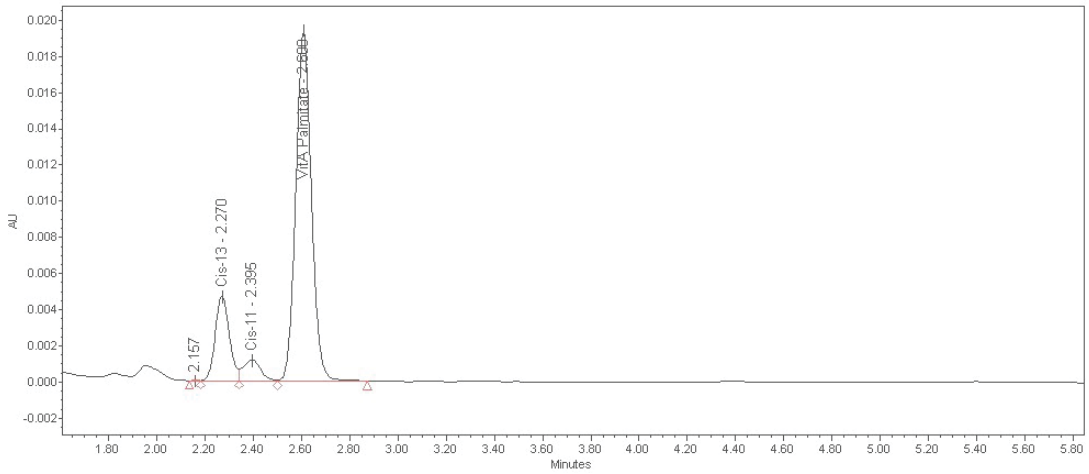

Figure 2012.10B. Vitamin A palmitate sample chromatograms.

$$IU/mL = \frac{Abs}{43.6} \times \frac{100}{3} \times 10$$

where *Abs* = average absorbance reading, determined above; 43.6 = extinction coefficient of tocopheryl acetate at 284 nm; and 10 = conversion of percent to mg/mL. (*Note*: For dl-α-tocopherol acetate, 1 mg = 1 IU.)

(**d**) dl-*α-Tocopherol*.—(*1*) Pipet 3 mL stock solution E2 into a l00 mL volumetric flask and make up to volume with iso-octane.

(*2*) Determine the absorption at 292 nm, zeroed against iso-octane in a 1 cm quartz cell.

(*3*) Repeat the reading twice, rinsing the sample cuvet with the solution before each reading. Calculate the average absorbance reading. The potency of the α-tocopherol stock solution is then calculated as follows:

$$IU/mL = \frac{Abs}{75.8} \times \frac{100}{3} \times 10 \times 1.1$$

where *Abs* = average absorbance reading, determined above; 75.8 = extinction coefficient of α-tocopherol at 292 nm; 10 = conversion of percent to mg/mL; and 1.1 = conversion from mg to IU for dl-α-tocopherol.

(**e**) *Calculation of working standard concentrations*.—The concentration of each vitamin in the working standards is calculated from the stock concentration using the appropriate dilution factor, as shown (IU/mL), Section **F**. Refer to the example calculations below.

(*1*) *Level 1 calibration standard*.

$$\text{Vitamin A Palmitate } (IU/mL) = Stock\ IU/mL \times \frac{4}{50} \times \frac{4}{50} \times \frac{0.5}{50} \times \frac{2}{10}$$

$$\text{Vitamin A Acetate } (IU/mL) = Stock\ IU/mL \times \frac{4}{50} \times \frac{4}{50} \times \frac{0.5}{50} \times \frac{2}{10}$$

$$DL-\alpha\ Tocopherol\ Acetate\ (IU/mL) = Stock\ IU/mL \times \frac{7}{50} \times \frac{4}{50} \times \frac{0.5}{50} \times \frac{2}{10}$$

$$DL-\alpha\ Tocopherol\ (IU/mL) = Stock\ IU/mL \times \frac{20}{50} \times \frac{4}{50} \times \frac{0.5}{50} \times \frac{2}{10}$$

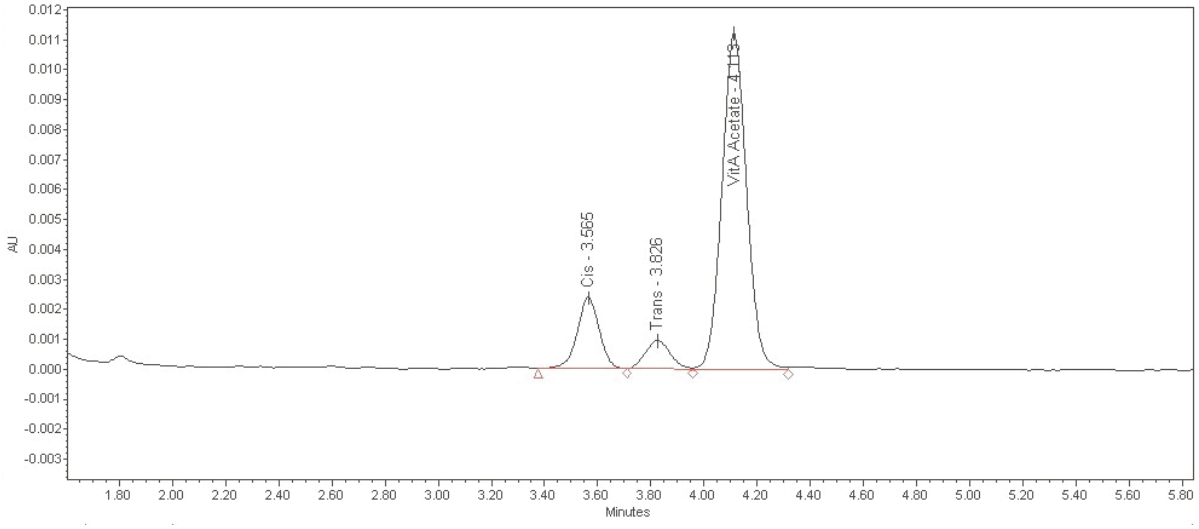

Figure 2012.10C. Vitamin A acetate sample chromatograms.

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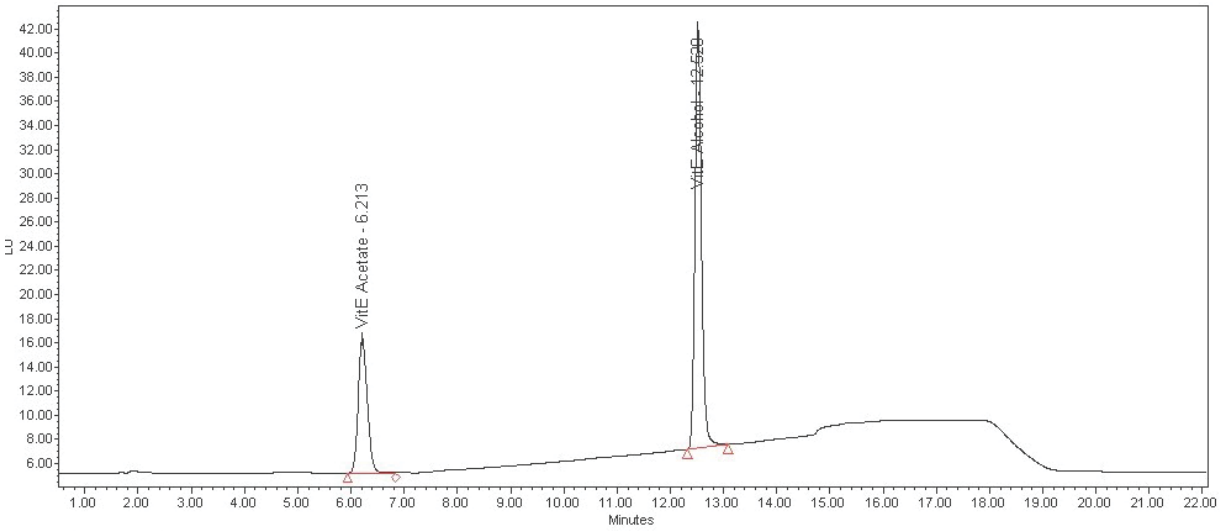

Figure 2012.10D. α-Tocopherol acetate and α-tocopherol calibration standard.

H. Sample Preparation

(**a**) For powder samples, transfer 25 g, accurately weighed, into a 250 mL volumetric flask. Dissolve using distilled water (approximately 40°C), cool and make up to 250 mL with distilled water. Transfer 5 mL reconstituted sample to a 50 mL screw top centrifuge tube.

(**b**) For ready-to-feed samples or concentrated liquid products, transfer 5.0 mL thoroughly agitated sample directly to a 50 mL screw top centrifuge tube. Liquid samples should be analyzed from a freshly opened container, stored refrigerated for no more than 48 h, and never analyzed from a frozen sample.

(**c**) Add 5 mL 2% papain solution.

(**d**) Mix to disperse each sample, cap, and place the tubes in a 37±2°C water bath for 20–25 min. Remove the samples from the bath and cool. Place in a freezer for approximately 5 min or refrigerate for approximately 20 min.

(**e**) Add approximately 20 mL acidified methanol to each sample tube and mix.

(**f**) Accurately pipet 10.0 mL iso-octane into each sample tube. Close tightly to avoid leakage and shake the tube for 10 min, preferably with a mechanical shaker.

(**g**) Centrifuge for 10 min at 4000 rpm to obtain a clear iso-octane layer. Remove enough iso-octane from the centrifuge tube to fill an injection vial. This extract is ready for LC analysis.

(**h**) Typically, a 50 µL injection volume is used for the standards and sample extracts, but this can be varied (20–100 µL) to suit sensitivity.

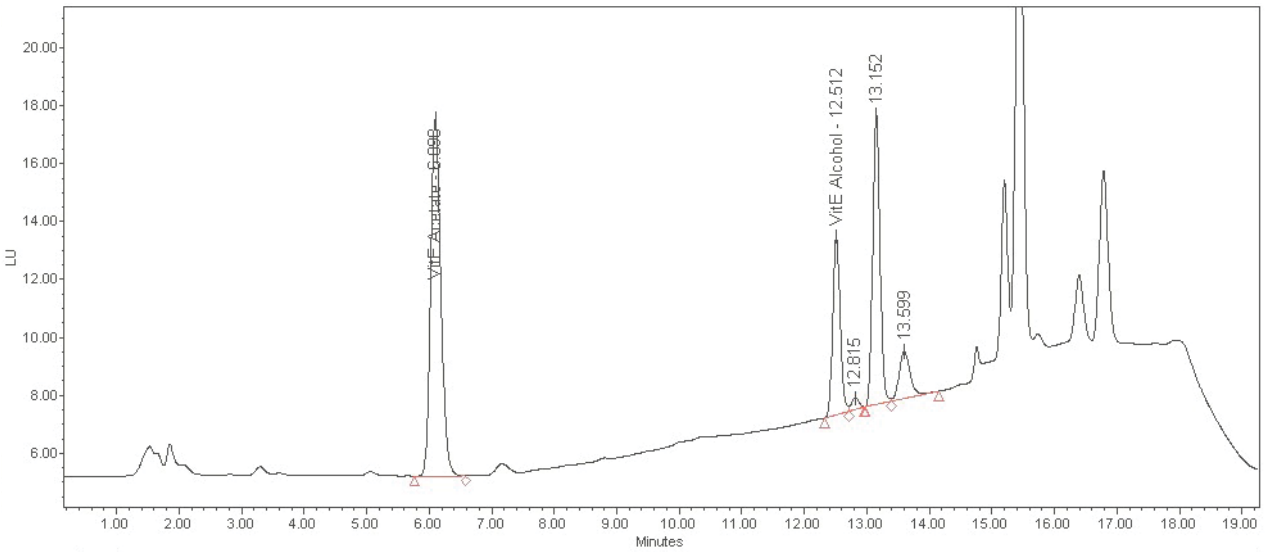

Figure 2012.10E. α-Tocopherol acetate and α-tocopherol sample chromatogram.

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I. HPLC Analysis

(**a**) Use the pump gradient elution cycle (Table **2012.10**).

(*Note*: The gradient parameters can be altered as required to maximize the analytical separation and avoid interferences.)

(**b**) Set the detectors to the following wavelengths: UV detector, 325 nm for vitamin A palmitate and vitamin A acetate. FLD (excitation/emission) 280/310 nm for dl-α-tocopherol acetate and dl-α-tocopherol (Figures **2012.10A–D**).

J. System Suitability

The following system suitability and standard checks are to be met when running this method.

(**a**) *Linearity*.—The coefficient of determination, R^2, of each calibration curve shall be ≥0.995.

(**b**) *Standard injection precision*.—When a stable baseline is obtained, inject a medium reference standard three times in succession and determine the RSD. The RSD determined must be ≤2%.

(**c**) *Standard response accuracy*.—Determine the slope change between successive calibrations and the difference must be ≤3%. The appropriate number of samples between successive calibrations is typically 6–8.

(**d**) *Tailing/asymmetry factor*.—The target limits of the tailing factor for meeting system suitability are 0.8–1.2. The system should be closely monitored if outside the target limits, and action taken if the value is outside 0.5–1.5 (e.g., column cleaning, reconditioning, or replacement).

K. Calculations of Sample Concentrations

(**a**) *Powder samples*.—

$$\textit{Vitamin Conc}\ (\textit{IU/100 g})\ \textit{reconstituted Final Product} = \frac{(\textit{Area/Height of Sample Peak} - C)}{\textit{Slope}} \times \frac{Y}{W} \times 10 \times 100 \times \frac{W}{225}$$

where C = y-intercept; Y = dilution volume of test portion; 10 = volume (mL) of iso-octane; 100 = conversion to per 100 g; W = sample amount in g; and 225 = weight of dilution water.

(**b**) *Liquid samples*.—

$$\textit{Vitamin Conc.}\ (\textit{IU/100 g reconstituted Final Product}) = \frac{(\textit{Area/Height of Sample Peak} - C)}{\textit{Slope}} \times \frac{Y}{W} \times 10 \times 100 \times D$$

where C=y-intercept, Y=dilution volume of test portion, 10=volume (mL) of iso-octane, 100=conversion to per 100 g, W=sample amount in mL for liquid ready-to-feed and concentrates, and D=density of liquid product. Total vitamin A is the sum of the *trans* vitamin A concentration and the 13-*cis* vitamin A concentration in IUs.

One IU is equal to 0.30 µg *all-trans* retinol. Retinyl palmitate or retinyl acetate in IU/100 g reconstituted final product can be converted to µg/100 g reconstituted final product in retinol equivalents (REs) by multiplying by 0.30.

One RE is defined as 1 µg retinol.

Total vitamin E (sum of dl-α-tocopherol and dl-α-tocopherol acetate) in IU/100 g reconstituted final product can be converted to mg/100 g reconstituted final product in α-TEs by multiplying by 0.671.

References: *J. AOAC Int*. **96**, 1073(2013)
DOI: 10.5740/jaoacint.13-103

AOAC SMPR 2011.003
J. AOAC Int. **95**, 291(2012)
DOI: 10.5740/jaoac.int.11-0439

AOAC SMPR 2011.010
J. AOAC Int. **96**, 485(2013)
DOI: 10.5740/jaoac.int.SMPR2011.010

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AOAC Official Method 2012.13
Determination of Labeled Fatty Acids Content in Milk Products and Infant Formula

Capillary Gas Chromatography
First Action 2012

A. Scope

The method involves the quantification of all labeled fatty acids that includes groups of fatty acids [i.e., *trans* fatty acids (TFA), conjugated linoleic acids (CLA), saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), omega-3, omega-6, omega-9] and/or individual fatty acids [i.e., linoleic acid (LA), α-linolenic acid (ALA), arachidonic acid (ARA), ecosapentaenoic acid (EPA), docosahexaenoic acid (DHA)] (*see* also **J**) in milk products and infant formula, containing milk fat and/or vegetable oils, supplemented or not supplemented with long-chain polyunsaturated fatty acids (LC-PUFA).

The determination is performed by direct transesterification of food matrixes, without prior fat extraction, and consequently it is applicable to liquid samples or reconstituted powders.

Products containing less than 1.5% fat can be analyzed after preliminary fat extraction using methods described.

In the case of products supplemented/enriched with PUFA having fish oil or algae origins, a cold fat extraction procedure is recommended (evaporation of solvents at lower temperature).

B. Principle

Addition of the internal standard solution to the sample, preparation of fatty acid methyl esters (FAMEs) by direct transesterification with methanolic sodium methoxide for liquid samples; dissolution in water and direct transesterification with methanolic sodium methoxide for powdered samples. Separation of FAMEs using capillary gas-liquid chromatography. Identification by comparison with the retention time of pure standards and quantification as fatty acids by reference to an internal standard. Verification of the transesterification performance using a second internal standard.

C. Apparatus

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; ISO class AS (ISO).

(**d**) *Two-marks pipet, volumetric, with two marks*.—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, 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.

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 will affect the chromatography duration especially, and lessen the resolution.

(*2*) *Other gases*.—Free from organic impurities (CnHm of below 1 ppm), nitrogen and hydrogen, purity at least ≥99.995%, and synthetic air.

(*3*) *Capillary column*.—With a stationary phase which has been successfully employed to perform FAMEs separation. It is suggested to use cyanopropyl-polysiloxane phase capillary columns (100 m length × 0.25 mm id, 0.25 µm film thickness) that elute the FAMEs, primarily by carbon chain length and secondarily by the number of double bonds.

Note: Traces of oxygen and humidity will damage the polar phase of the column. If 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, and distilled or demineralized water or water of equivalent purity.

(**a**) *Water*.—HPLC grade.

(**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 make up 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. This solution volume is sufficient to analyze ca 40 samples. In case of smaller number of analysis, reagent volume can be adapted accordingly.

Perform the transesterification reaction at ambient temperature (between 20 to 25°C).

(**d**) *Disodium hydrogen citrate sesquihydrate [$HOC(COOH)(CH_2COONa)_2 \cdot 1.5H_2O$]*.

(**e**) *Neutralization solution*.—Disodium hydrogen citrate sesquihydrate 10%, sodium chloride 15% in water.

Weigh 50.0 g disodium hydrogen citrate sesquihydrate and 75.0 g sodium chloride in a 500 mL volumetric flask, **C**(**b**). Dissolve in 450 mL water using a magnetic stirrer. Remove the magnetic stirrer, then make up to the mark with water.

Stored in the dark at 4°C, this solution is stable for 1 month. Presence of salt crystals may appear in the solution during storage, but disappear after shaking.

Allow the solution to come to room temperature before use. This solution volume is sufficient to analyze ca 40 samples or more. In case of a smaller number of analysis (or single analysis), weights and volume of solution can be adapted accordingly.

(**f**) *tert-Butyl methyl ether (MTBE)*.

(**g**) *Methyl undecanoate (C11:0 FAME)*.—Purity ≥99% mass fraction.

(**h**) *Tritridecanoin (C13:0 TAG)*.—Purity ≥99 % mass fraction.

(**i**) *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 and 500 mg methyl undecanoate. Dissolve and make up to the mark with MTBE.

Stored in the dark at 4°C, this solution is stable for 1 week. Allow the solution to come to room temperature before use.

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This solution volume is sufficient to analyze ca 40 samples or more. In case of a smaller number of analysis, standard weights and volume of solvent can be adapted accordingly.

(**j**) *Octadecenoic acid methyl ester*.—*cis/trans*-Isomers 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 Sigma-Aldrich (Cat. No. 40495-U).

(**k**) *Linoleic acid methyl ester*.—*cis/trans*-Isomers mixture of C18:2 n-6 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: Standard commercially available from Supelco Inc. (Cat. No. 47791; Supelco Inc., or equivalent, is an example of suitable product available commercially).

(**l**) *Linolenic acid methyl ester*.—*cis/trans*-Isomer mixture of C18:3 n-6 with *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid methyl ester ~3% (w/w), *cis*-9,*cis*-12,*trans*-15-octadecatrienoic acid methyl ester ~7% (w/w), *cis*-9,*trans*-12,*cis*-15-octadecatrienoic acid methyl ester ~7% (w/w), *cis*-9,*trans*-12,*trans*-15-octadecatrienoic acid methyl ester ~15% (w/w), *trans*-9,*cis*-12,*cis*-15-octadecatrienoic acid methyl ester ~7% (w/w), *trans*-9,*cis*-12,*trans*-15-octadecatrienoic acid methyl ester ~15% (w/w), *trans*-9,*trans*-12,*cis*-15-octadecatrienoic acid methyl ester ~15% (w/w), and *trans*-9,*trans*-12,*trans*-15-octadecatrienoic acid methyl ester ~30% (w/w). Concentration 10 mg/mL in methylene chloride. This standard is commercially available from Supelco Inc. (Cat. No. 47792).

Note: This standard contains all *trans* isomers but their abundance and ratio are different from those observed in oils and fats.

(**m**) *Methyl octadecadienoate conjugated acids*.—Mixture of C18:2 *cis*-9,*trans*-11 and *cis*-10,*trans*-12; purity ≥99%.

(**n**) *Qualitative cis/trans FAME isomers standard mixture solution*.—For the RT identification of *cis/trans* isomers, prepare a qualitative standard solution with the standard listed, **D**(**l**)–(**n**). All standards commercially available could be used. Into a 50 mL volumetric flask, add each standard isomer in equal proportion. Dissolve and make up to the mark with hexane. Dilute according to the type of injector used.

(**o**) *Standard FAME mixture solution*.

(*1*) Purchase individual FAME standard list as follows (purity ≥99%): 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*), heneicosanoic acid methyl ester (C21:0), *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*), *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester (C22:6 n-3 *cis*).

Note: The purchasing of individual FAME standards is more expensive in comparison to FAME standard mixture. In addition the individual weight for each FAME standards could give imprecision.

(*2*) Purchase a quantitative FAME standard mixture: Nu-Check-Prep, Cat. No. GLC- Nestle-36 (Nu-Check-Prep GLC-Nestle36, or equivalent).

The FAME calibration standard mixture is carefully prepared by weight by the supplier. The weight percentage of each 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.

(**p**) *Home-made calibration standard FAME mixture solution.*

(*1*) *Stock solution 1: Saturated.*—Into a 100 mL volumetric flask, accurately weigh ca 25 mg lignoceric acid methyl ester (C24:0), 25 mg behenic acid methyl ester (C22:0), 25 mg heneicosanoic acid methyl ester (C21:0), 25 mg arachidic acid methyl ester (C20:0), 25 mg stearic acid methyl ester (C18:0), 25 mg heptadecanoic acid methyl ester (C17:0), 50 mg palmitic acid methyl ester (C16:0), 25 mg pentadecanoic acid methyl ester (C15:0), 25 mg myristic acid methyl ester (C14:0), 25 mg tridecanoic acid methyl ester (C13:0), 25 mg lauric acid methyl ester (C12:0), 25 mg undecanoic acid methyl ester (C11:0), 25 mg capric acid methyl ester (C10:0), 25 mg caprylic acid methyl ester (C8:0), 25 mg caproic acid methyl ester (C6:0), and 25 mg butyric acid methyl ester (C4:0). Make up to the mark with *n*-hexane.

Note: Palmitic acid is weighed in double amount.

(*2*) *Stock solution 2: Monounsaturated.*—Into a 100 mL volumetric flask, accurately weigh ca 25 mg nervonic acid methyl ester (C24:1 n-9 *cis*), 25 mg erucic acid methyl ester (C22:1 n-9 *cis*), 25 mg *cis*-11-eicosenoic acid methyl ester (C20:1 n-9 *cis*), 25 mg oleic acid methyl ester (C18:1 n-9 *cis*), 25 mg elaidic acid methyl ester (C18:1 n-9 *trans*), 25 mg *cis*-10-heptadecenoic acid methyl ester (C17:1 n-7 *cis*), 25 mg palmitoleic acid methyl ester (C16:1 n-7 *cis*), 25 mg *cis*-10-pentadecenoic acid methyl ester (C15:1 n-5 *cis*), and 25 mg myristoleic acid methyl ester (C14:1 n-5 *cis*). Make up to the mark with *n*-hexane.

(*3*) *Stock solution 3: Polyunsaturated.*—Into a 100 mL volumetric flask, accurately weigh ca 25 mg linolelaidic acid methyl ester (C18:2 n-6 *trans*), 25 mg linoleic acid methyl ester (C18:2 n-6 *cis*), 25 mg gamma-linoleic acid methyl ester (C18:3 n-6 gamma), 25 mg linolenic acid methyl ester (C18:3 n-3 *cis*), 25 mg *cis*-11,14-eicosadienoic acid methyl ester (C20:2 n-6 *cis*), 25 mg *cis*-8,11,14-eicosatrienoic acid methyl ester (C20:3 n-6 *cis*), 25 mg *cis*-11,14,17-eicosatrienoic acid methyl ester (C20:3 n-3 *cis*), 50 mg arachidonic acid methyl ester (C20:4 n-6 *cis*), 25 mg *cis*-13,16-docosadienoic acid methyl ester (C22:2 n-6 *cis*), 25 mg *cis*-5,8,11,14,17-eicosapentanoic acid methyl ester (C20:5 n-3 *cis*), and 25 mg *cis*-4,7,10,13,16,19-docosahexaenoic acid methyl ester (C22:6 n-3 *cis*). Make up to the mark with *n*-hexane.

(*4*) *Diluted calibration standard solution.*—Into a 100 mL volumetric flask pipet 25.0 mL of the calibration standard stock solution 1, **D**(**p**)(*1*), 25.0 mL of the calibration standard stock

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solution 2, **D**(**p**)(*2*), and 25.0 mL of the calibration standard stock solution 3, **D**(**p**)(***3***). Then make up to the mark with *n*-hexane. Dilute accordingly to the type of injector used.

Note: This solution keeps for about 6 months if stored in the dark at –20°C. To prevent contamination of the standard solution, immediately distribute the solution into different vials (ready to inject) and store at –20°C before use. Use each vial once then discard it.

(**q**) *Commercially available calibration standard FAME mixture solution*.—Before use, allow the ampoule to come to room temperature (maximum 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 pre-tarred volumetric flask, weigh, and make up to the mark with *n*-hexane. Dilute accordingly to the type of injector used.

Note: This solution keeps for about 6 months if stored in the dark at –20°C. To prevent contamination of the standard solution, immediately distribute the solution into different vials (ready to inject) and store at –20°C before use. Use each vial once and then discard it.

E. Preparation of Test Sample

Milk powder and infant formula.—Mix well to ensure that sample is homogeneous.

(*1*) *Class or group of fatty acids in 100 g fat.*—Calculate the mass fraction of all fatty acids included in a group or in a class of fatty acids by simple addition of individual fatty acids results (expressed in g FA/100 g fat).

(*2*) *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 in %, 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 C-11:0 internal standard added to the solution; A_{C13} is the peak area of C-13:0 internal standard in the chromatogram; R_{C13} is the response factor of C13:0 relative to C11:0, calculated according to **G**(**a**)(*2*); S_{C13} is the stoichiometric factor to convert C13:0 FAME into C13:0 TAG; A_{C11} is the peak area of C-11:0 internal standard in the chromatogram; and m_{C13} is the mass of liquid milk and liquid formula. Bring sample to room temperature and shake vigorously before use.

F. Procedure

As a check on method performance, carry out two single determinations in accordance with **F**(**a**) and (**b**).

Note: An alternative procedure using fat-extraction tubes with siphon or wash-bottle fittings is given in Figures **2012.13A–C**.

(**a**) *Resolution between C18:1 trans and cis*.—Inject once the qualitative *cis/trans* FAME isomers standard mixture solution, **D**(**o**).

(**b**) *Calibrating solution for the determination of response factor*.—Inject three times the calibrated solution, **D**(**q**).

(**c**) *Test portion*.—Into a 25 mL centrifuge tube with a screw cap, weigh to the nearest 0.1 mg an equivalent quantity of sample in order to have 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 1: For fatty acid analysis on fat extracted from foods, the same amount of fat is required. 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 2: For liquid milk samples and fat extracted from foods, no pretreatment (water addition) is required.

Pipet 5 mL of internal standard solution, **D**(**j**). Add with a pipet 5 mL of 5% (w/v) methanolic sodium methoxide solution, **D**(**d**). The transesterification time starts with the addition of the first drop. 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. Shake gently using a vortex mixer. The transesterification time should not exceed 240 s.

Note 3: To respect the total reaction time (240 s), the transesterification reaction should be carried out with a maximum of six tubes at the same time. Rapid delivery system (dispenser) can be used to add reagents, but not for the addition of internal standard solution.

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 of the supernatant and make up to the mark with *n*-hexane.

Note 4: The dilution factor is calculated for on-column and splitless injection only. When using split injection, adapt the dilution to obtain the desired peak responses accordingly 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.

G. Determination

(**a**) *Quantitative determination.*

(*1*) *Resolution.*—Inject into the gas chromatograph 1.0 µL of the calibrating solution, **F**(**a**). Determine peak width at half height and distance between the left of the chromatogram and the top of peak for C18:1 *trans* Δ13/14 and oleic acid methyl ester. The resolution criteria (R) is calculated as follows:

$$R = 1.18(t_{R2} - t_{R1})/W_{(1/2)1} + W_{(1/2)2}$$

where t_{R1} = distance, in centimeters, between the left of the chromatogram and the top of peak 1 (C18:1 *trans* Δ13/14); t_{R2} = distance, in centimeters, between the left of the chromatogram and the top of peak 2 (oleic acid); $W_{(½)1}$ = peak width, in centimeters, at half height of peak 1 (C18:1 *trans* Δ13/14); $W_{(½)2}$ = peak width, in centimeters, at half height of peak 2 (oleic acid).

The resolution test is acceptable when value calculated for R is equivalent or higher than 1.00 (±5%) (to determine with the ring test).

Note: Example of the calculation is given in Figure **2012.13C**.

(*2*) *Calculation of response factor.*—Inject into the gas chromatograph 1.0 µL of the calibrating solution, **F**(**b**). Determine the area of peaks attributable to each FAME present in the calibration standard mixture, **D**(**q**), and calculate their respective response factors (Rf_i) relative to the internal standard (C11:0):

$$Rf_i = \frac{m'_i \cdot A'_O}{m'_O \cdot A'_i}$$

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where m'_i = mass fraction of $FAME_i$ in the calibration standard solution, **D(q)**; 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(q)**; 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 less than 2.

Note: The response factors calculated for C18:2 n-6 could be applied for C-18:2 CLA and that calculated for C18:3 n-3 *cis* could be applied for C18:3 *trans* isomers.

(*3*) *Determination of the test portion*.—Inject 1 µL of the test portion, **F(b)**, into the gas chromatograph applying the same conditions as used in the calibrating solution.

(**b**) *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)**.

C18:1 TFA.—Identify and group all TFA of C18:1 (include also the peak area of *trans* C18:1 *trans* Δ16 eluted in the *cis* C18:1 region just after the oleic acid) according to Figures **2012.13A** and **B**.

Note: When milk fat is present, two trans isomers of C18:1 are eluted in the *cis* C18:1 region (the C18:1 *trans* Δ15 and C18:1 *trans* Δ16, respectively), but only one (C18:1 *trans* Δ16) is resolved with the 100 m length capillary column. The second isomer (C18:1 *trans* Δ15) is generally overlapped with the oleic acid peak (C18:1 *cis* Δ9) and its area is only quantifiable using a preliminary separation (TLC Ag^+, HPLC Ag^+) followed by a capillary GLC analysis. According to recent findings, it has been demonstrated that there is not significant difference of total C18:1 *trans* amount when the area of C18:1 *trans* Δ15 (not resolved peak) is not included in the sum in comparison to the result obtained after preliminary separation techniques (Ag^+) followed by a capillary GLC analysis. A part of this phenomenon is explained by presence of some C18:1 *cis* isomers (Δ6-8) which elute in the C18:1 *trans* and consequently are indirectly added to the sum of C18:1 *trans* and compensate the fact that C18:1 *trans* Δ15 is not taken into account.

C18:2 TFA.—Identify and group all TFA of linoleic (C18:2 n-6) acids (*see* Figures **2012.13A** and **B**). For the total TFA of C18:2, include all the *trans* isomers present in milk fat sample as shown in Figures **2012.13A** and **B**.

C18:3 TFA.—Identify and group all TFA of linolenic (C18:3 n-3) acids (*see* Figures **2012.13A** and **B**).

Note: In presence of milk fat and/or fish oil in the sample, another isomer of C20:1 elutes just before C20:1 n-9. Depending on the column resolution, the retention time of this fatty acid may also correspond to a *trans* isomer of C18:3 n-3 (the c,t,c or t,c,c). 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 below). Interferences could be also observed between C18:3 TFA isomers (C18:3 c,c,t; c,t,c; or t,c,c) and C20:1 n-9. When C20:1 n-9 elute with C18:3 c,t,c, (the minor C18:3 TFA isomer), their contribution on the total C18:3 TFA is negligible. However, if C20:1 n-9 is interfered with C18:3 c,c,t or with C18:3 t,c,c the chromatography conditions should be slightly modified to obtain sufficient separation. Interference is also visible when wrong ratio between C18:3 n-3 c,c,t and C18:3 n-3 t,c,c is observed (normal ratio is always 5:4).

The kinetics of C18:3 *trans* isomers formation in refined and deodorized oils have been analyzed using highly polar capillary column and described in the literature. They could be used as a

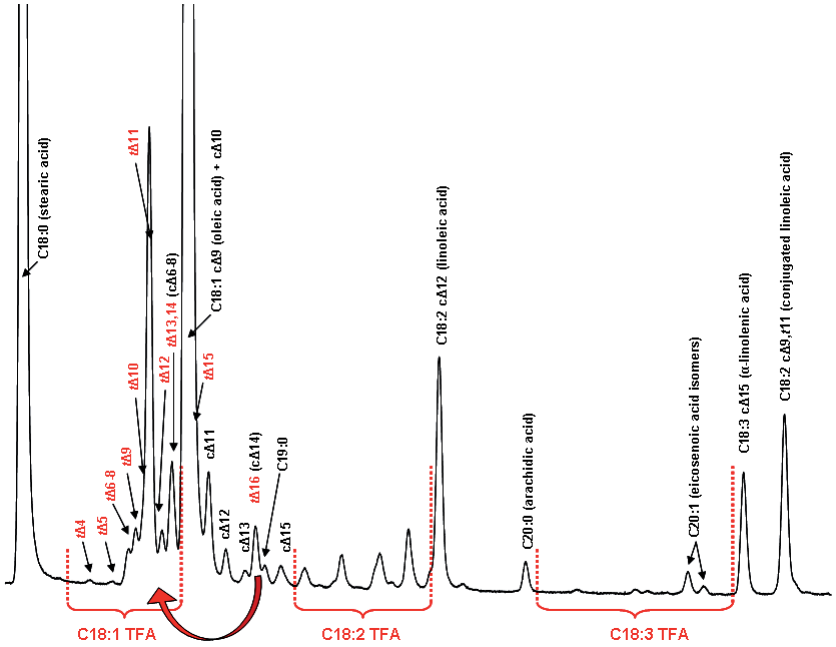

Figure 2012.13A. Example of GC chromatogram (enlarged view of C18:1 TFA, C18:2 TFA, and CLA) using split injection.

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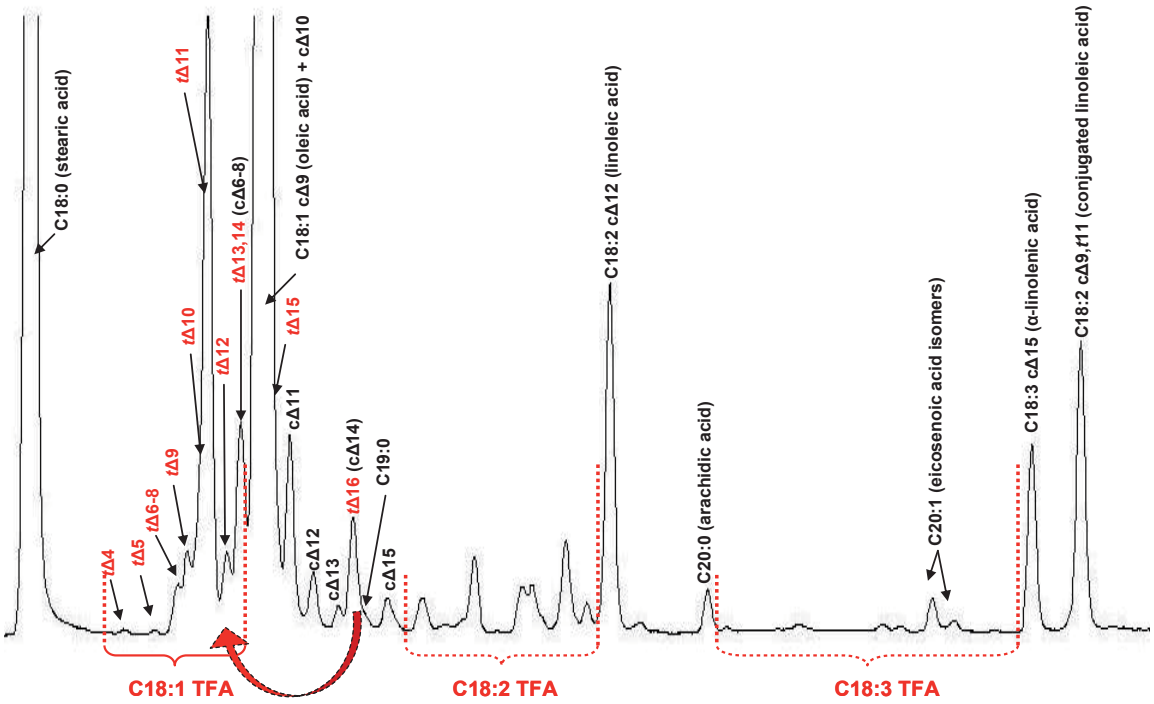

Figure 2012.13B. Example of chromatogram (enlarged view of C18:1 TFA, C18:2 TFA, C18:3 TFA, and CLA) using on-column injection.

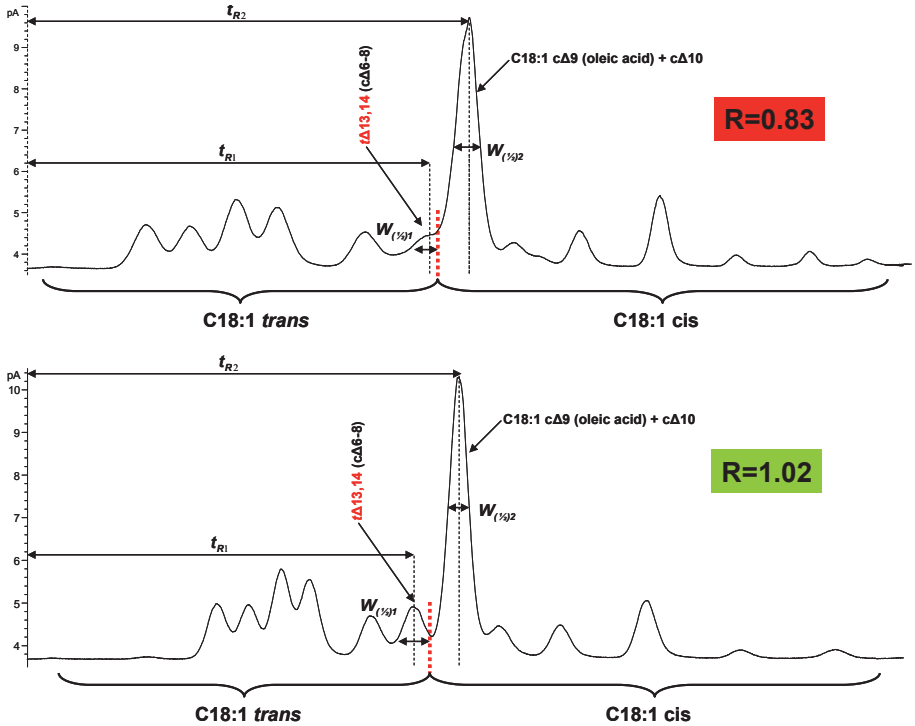

Figure 2012.13C. Example of GC chromatogram (insufficient and sufficient resolution for C18:1 *trans*).

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Table 2012.13A. Stoichiometric factors (Si FA) for converting the fatty acid methyl esters (FAME) to fatty acids (FA)

Chain length	Configuration and group			Abbreviation	FAME m.w.	FA m.w.	TAG m.w.	Si (FA)
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		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*[a]			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*[a]			294.5	280.5	879.4	0.952
C18:2	ω-6 (or n-6)	*cis*	PUFA	LA	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*[a]			292.5	278.4	873.4	0.952
C18:3	ω-3 (or n-3)	*cis*	PUFA	ALA	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	318.5	304.5	951.5	0.956
C20:5	ω-3 (or n-3)	*cis*	PUFA	EPA	316.5	302.5	945.4	0.956
C21:0			SFA		340.6	326.6	1017.8	0.959
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	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] Does not include trans fatty acids into MUFA and PUFA sums.

confirmatory tool to verify the presence of TFA isomers. Most of the time, a maximum number of four isomers is encountered.

Case 1.—Absence of C18:3 TFA isomers.—No peak (if only one peak is detected; *see* the comment above regarding the presence of another C20:1 isomer in milk). The presence of only one C18:3 TFA isomer is not possible.

Case 2.—Presence of C18:3 TFA isomers (a minimum of two isomers, C18:3 c,c,t and C18:3 t,c,c.).—The C18:3 t,c,c peak area is approximately 80% of the C18:3 c,c,t peak area (ratio 5:4). This ratio is always constant when other C18:3 TFA isomers are present.

Case 3.—Presence of C18:3 TFA isomers (three isomers, C18:3 c,c,t; C18:3 c,t,c; and C18:3 t,c,c).—The same as described above for Case 2, but with the C18:3 c,t,c. The peak area of this isomer is always small and is sometime lower than the LOQ. In case of co-elution with C20:1 n-9 (or another C20:1 isomer), its contribution on total C18:3 TFA is negligible.

Case 4.—Presence of C18:3 TFA isomers (four isomers, C18:3 t,c,t; C18:3 c,c,t; C18:3 c,t,c; and C18:3 t,c,c).—The same as described above (Cases 2 and 3), but with the C18:3 t,c,t. This isomer is formed by the degradation of C18:3 c,c,t and C18:3

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Table 2012.13B. Calculation of response factors (example using Excel)

		Injection No.	1	2	3		
		File name	a	b	c	Response factor related to	
	Concn, w/v (%)	Injection date	xx-xx-xx	xx-xx-xx	xx-xx-xx		RSD, %
No.	2.70	Internal standard area	28547	28252	29071	C-11:0	(≤2.5)
1	2.70	C-4:0	17079	16985	17418	1.668	0.2
2	2.70	C-6:0	22310	22097	22560	1.282	0.4
3	2.70	C-8:0	25379	25106	26139	1.121	0.7
4	2.70	C-10:0	28170	27547	28612	1.018	0.6
5	2.70	C-11:0	28547	28252	29071	1.000	0.0
6	2.70	C-12:0	29670	29445	30392	0.959	0.3
7	2.70	C-13:0	30248	30049	30975	0.941	0.3
8	2.70	C-14:0	30949	30951	31576	0.919	0.6
9	2.70	C-14:1 n-5	30152	30262	31032	0.939	0.7
10	2.70	C-15:0	31022	31047	31828	0.915	0.6
11	2.70	C-15:1 n-5	30496	30660	31439	0.927	0.8
12	5.40	C-16:0	63406	63475	65161	0.894	0.6
13	2.70	C-16:1 n-7 *cis*	31064	30958	32114	0.912	0.8
14	2.70	C-17:0	31933	31946	32903	0.887	0.7
15	2.70	C-17:1 n-7	31734	31407	32676	0.896	0.6
16	2.70	C-18:0	32780	32259	33657	0.870	0.7
17	2.70	C-18:1 n-9 *trans*	32349	31876	33238	0.881	0.7
18	2.70	C-18:1 n-9 *cis*	32838	32334	33716	0.868	0.7
19	2.70	C-18:2 n-6 *trans*	32462	32053	33263	0.878	0.4
20	2.70	C-18:2 n-6 *cis*	32819	32440	33870	0.866	0.8
21	2.70	C-18:3 n-6 gamma	32256	32050	33135	0.881	0.4
22	2.70	C-18:3 n-3 alpha	32760	32594	33494	0.869	0.3
23	2.70	C-20:0	33158	32934	34113	0.857	0.5
24	2.70	C-20:1 n-9	32982	32781	33730	0.863	0.2
25	2.70	C-20:2 n-6	33128	32806	33750	0.861	0.0
26	2.70	C-20:3 n-6 DHGLA	32793	32611	33545	0.868	0.3
27	2.70	C-20:3 n-3	32674	32618	33653	0.868	0.6
28	2.70	C-20:4 n-6	32714	32590	33807	0.866	0.7
29	2.70	C-20:5 n-3 EPA	32005	31810	32963	0.887	0.6
30	2.70	C-21:0	33256	32906	33958	0.858	0.2
31	2.70	C-22:0	33265	33132	34129	0.854	0.4
32	2.70	C-22:1 n-9	32654	32628	33445	0.870	0.5
33	2.70	C-22:2 n-6	33227	33064	34259	0.854	0.6
34	2.70	C-22:6 n-3 DHA	32483	32264	33371	0.875	0.4
35	2.70	C-24:0	33817	33838	34712	0.839	0.6
36	2.70	C-24:1 n-9	32699	32464	33633	0.869	0.5

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t,c,c (the two principal C18:3 TFA isomers). When its amount is high, the presence of other C18:3 TFA isomers could be suspected possibly. The presence of other C18:3 isomers can be confirmed with the qualitative standard mixture, **D**(**o**).

Use the following terms to express TFA results:

C18:1 TFA.—The sum of *trans* positional isomers from C18:1.

C18:2 TFA.—The sum of *trans* isomers from C18:2 n-6 in deodorized oils (tt, ct, and tc) and in milk fat (C18:2 c9,t13 + C18:2 t8,c12 and C18:2 t11,c15).

C18:3 TFA.—The sum of *trans* isomers from C18:3 n-3 in deodorized vegetable oils (tct, cct, ctc, and tcc).

Total TFA.—Sum of C18:1 TFA, C18:2 TFA, and C18:3 TFA.

H. Gas Chromatographic Conditions

The oven temperature and the carrier gas flow depend on the column selected, and on the carrier gas adopted. In any case, the selected conditions shall produce the separation between *cis* and *trans* zone for C18:1, C18:2, C18:3, and CLA (Figures **2012.13A** and **B**).

For the accurate quantification of C18:1 TFA (level ≥ 0.5 g/100 g fat), a sufficient resolution between C18:1 *trans* Δ13/14 and oleic acid (C18:1 Δ9 *cis*) is required. The resolution is determined with the injection of the qualitative *cis/trans* C18:1 FAME isomers standard mixture solution, **D**(**o**). The resolution is sufficient when R criteria is equivalent or higher than 1.000 (Figure **2012.13C**).

The examples listed below report applicable conditions for a correct separation of *cis* and *trans*.

(**a**) *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.

(*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*.—250°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**.

(**b**) *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.

(*3*) *Carrier gas type*.—Hydrogen.

(*4*) *Column head carrier gas pressure*.—210 KPa (175–225 KPa).

(*5*) *Injector temperature*.—Cold.

(*6*) *Detector temperature*.—280°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**.

(**c**) *Flame ionization detector*.—Capable of being heated to a temperature 50°C above the final temperature of the column oven.

(**d**) *Split/splitless injector*.—Capable of being heated to a temperature 30°C above the final temperature of the column oven.

(**e**) *On-column injector*.

(**f**) *Injection syringe*.—10 µL.

(**g**) *Integration system*.—Preferably being computerized.

I. Calculation and Expression of Results

(**a**) *Calculation*.

(*1*) *Fatty acids on the product*.—Calculate the mass fraction of the individual components expressed in g FA_i/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 of C11:0 internal standard, in milligrams, added to the sample solution; A_i = peak area of $FAME_i$ in the sample chromatogram; RF_i = response factor, calculated according to **F**(**c**); S_i(FA) = stoichiometric factor to convert $FAME_i$ to FA_i (Tables **2012.13A** and **B**); A_O = peak area of C11:0 internal standard in the sample chromatogram; and m = mass of test portion, in milligrams.

Note 1: The response factors RF_i for C18:2 n-6 *cis* can be used for C18:2 CLA and the response factor RF_i for C18:3 n-3 *cis* can be used for C18:3 *trans* isomers.

Note 2: In 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 product. Consequently fatty acids results are expressed in g FA/100 g fat and not in g FA/100 g product with this equation. Results obtained in g FA/100 g fat could be then converted into g FA/100 g product with the fat extraction value determined with an appropriate validated extraction method. The declared fat value should not be used for the expression of fatty acids on finished products.

(*2*) *Fatty acids on the total fat*.—Calculate the mass fraction of the individual components expressed in g FA_i/100 g fat in test sample by using the following equation:

$$gFA_i\,/100g\,fat = \frac{gFA_i\,/100g\,product \cdot 100}{\%Fat}$$

This calculation can be only performed when the fat content is determined with an appropriate validated extraction method. Do not use the declared fat value for the expression of fatty acids on finished products.

(*3*) *Sum of class or group of fatty acids in 100 g product*.—Calculate the mass fraction of all fatty acids included in a group or in a class of fatty acids by simple addition of individual fatty acids results (expressed in g FA/100 g product).

Sum of, in millgrams, of C-13:0 internal standard added to the solution

The difference in recovery between the blank and the sample (or the reference sample) should not exceed 1.0% of the mean of the duplicate determinations.

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

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could be the following: Incomplete transesterification, partial degradation of one internal standard, or matrix effect.

J. Annex A: Groups or Class of Fatty Acids and Individual Fatty Acids

(**a**) *Group or class of fatty acid*.

(*1*) *Trans fatty acids (TFA)*.—TFA is the sum of fatty acids containing one or more nonconjugated double bonds in *trans* configuration (only C18:1, C18:2, and C18:3 *trans* are included in the sum).

Note: Presence of other *trans* isomers naturally present in milk fat have been reported in literature (e.g., C16:1), but their contributions have no significant influence on the total amount of *trans* in milk products. In addition, their identification are complex especially because these isomers are frequently interfered with other isomers (e.g., *cis*, *iso*, and *anteiso*) and need preliminary separation technique.

(*2*) *Conjugated linoleic acids (CLA)*.—CLA are the sum of octadecadienoic acids containing conjugated double bonds in *cis* or *trans* configuration; mainly *cis*-9, *trans*-11 octadecadienoic acid (i.e., rumenic acid).

(*3*) *Saturated fatty acids (SFA)*.—SFA are the sum of all fatty acids which contain no double bonds.

(*4*) *Monounsaturated fatty acids (MUFA)*.—MUFA are the sum of all fatty acids containing one double bonds in *cis* configuration.

(*5*) *Polyunsaturated fatty acids (PUFA)*.—PUFA are the sum of all fatty acids containing two or more double bonds in *cis* configuration.

Note: LC-PUFA is the generic name to describe the long-chain polyunsaturated fatty acids, but these fatty acids are also included into PUFA (i.e., arachidonic, ecosapentaenoic, and docosahexaenoic acids).

(*6*) *Omega-3 fatty acid (ω-3)*.—Omega-3 fatty acid is the sum of *cis* PUFA having the first double bonds at carbon (n-3) from the terminal methyl group.

(*7*) *Omega-6 fatty acid (ω-6)*.—Omega-6 fatty acid is the sum of *cis* PUFA having the first double bonds at carbon (n-6) from the terminal methyl group.

(*8*) *Omega-9 fatty acid (ω-9)*.—Omega-3 fatty acid is the sum of *cis* PUFA having the first double bonds at carbon (n-9) from the terminal methyl group.

(**b**) *Individual fatty acids*.

(*1*) *Linoleic acid (LA)*.—LA is an essential fatty acid; an 18 carbon fatty acid containing two double bonds at carbons 9 and 12.

(*2*) *Linolenic acid (ALA) also called α-linolenic acid*.—ALA is an essential fatty acid; an 18 carbon fatty acid containing three double bonds at carbons 9, 12, and 15.

(*3*) *Arachidonic acid (ARA)*.—ARA is not an essential fatty acid; a 20 carbon fatty acid containing four double bonds at carbons 5, 8, 11, and 14.

(*4*) *Ecosapentaenoic acid (EPA)*.—EPA is a semi-essential fatty acid (essential for pregnant woman and infant); a 20 carbon fatty acid containing five double bonds at carbons 5, 8, 11, 14, and 17.

(*5*) *Docosahexaenoic acid (DHA)*.—DHA is a semi-essential fatty acid (essential for pregnant woman and infant); a 22 carbon fatty acid containing six double bonds at carbons 4, 7, 10, 13, 16, and 19.

Reference: *J. AOAC Int*. (future issue)

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STAKEHOLDER PANEL ON INFANT FORMULA AND ADULT NUTRITIONALS (SPIFAN)

Meeting held at

Boca Raton Resort & Club

Tuesday, September 9, 2014 - 8:30am (Eastern US)

REPORT OF THE EXPERT REVIEW PANEL (ERP) PROCEEDINGS

Expert Review Panel Members (in attendance):

Darryl Sullivan	***Covance Labs*** *(Chair)*
John Austad	Covance Labs
Sneh Bhandari	Mérieux NutriSciences & OMB
Esther Campos-Gimenez/Adrienne McMahon	Nestlé/Wyeth Nutrition (formerly Pfizer)
Scott Christiansen	Perrigo/PBM Nutritionals
Jon DeVries	General Mills/Medallion Labs
Brendon Gill/Harvey Indyk	Fonterra
Sarwar Gilani	Consultant
Min Huang	Frontage Labs, Inc.
Estela Kneeteman	INTI
Maria Ofitserova	Pickering Labs, Inc.
Melissa Phillips/Kate Rimmer	NIST
Shay Phillips	Mead Johnson Nutrition
Guenther Raffler	CLF-Eurofins
Karen Schimpf	Abbott Nutrition
Scott Christiansen	Perrigo/PBM Nutritionals
Jinchuan Yang	Waters Corp.

Expert Review Panel Members (unable to attend):

Don Gilliland	Abbott Nutrition
William Mindak	FDA
Jeanne Rader	FDA (CFSAN) - Retired

AOAC Staff Includes:

Delia Boyd
E. James Bradford
Scott Coates
Deborah McKenzie
Tien Milor
Anita Mishra
Bob Rathbone

Observers:

Martin Alewijn, *RIKILT*
Sean Austin, *Nestlé Research Center*
Brad Barrett, *ABSciex*
Christopher J. Blake, *Nestlé Research Center*
Martin Bucknall, *UNSW*
Marti Cenky, *Abbott Nutrition*
France Cho, *Maxxam Analytics Inc.*
Mark Collison, *Archer Daniels Midland Co.*
Hans Cruijsen, *FrieslandCampina Domo*
Brian De Borba, *Thermo Fisher Scientific*
Jean-Luc Deborde, *SCL Laboratoire de Strasburg*
Rachel de Guzman, *Mead Johnson Nutrition*
Marieke de Laat, *Mead Johnson Nutrition*
XiaoJun Deng, *SHCIQ*
Marcel deVreeze, *NEW/ISO*
Aurélie Dubois-Lozier, *IDF*
Wayne C. Ellefson, *Covance Laboratories*
Jaap Evers, *ISO Rep. (Fonterra Co-op.)*
Ping Feng, *Consultant*
Jennifer Fruth, *Mead Johnson Nutrition*
Pierre-Alain Golay, *Nestlé Research Center*
Phillip Haselberger, *Abbott Nutrition*
Melissa Holskey, *Abbott Nutrition*
Steve Holroyd, *IDF Rep. (Fonterra Co-op.)*
Gregory Hostetler, *Perrigo/PBM Nutritionals*
Wesley Jacobs, *Abbott Nutrition*
Greg Jaudzems, *Nestlé*
Elaine Jobgen, *Eurofins*
George Joseph, *AsureQuality*
Bert Klarenbeek, *FrieslandCampina Domo*
Khammawan Kohler, *Covance Laboratories*
Erik J. M. Konings, *Nestlé Research Center*
Li Xian Liang, *China ChongQing CIQ*
Stephen Lock, *ABSciex*
E. Marley, *R-Biopharm Rhome Ltd. Scotland*
Frederic Martin, *Nestlé Research Center*
Josh Messerly, *Eurofins*
Deepali Mohindra, *Thermo Fisher Scientific*
Nancy Montgomery, *Abbott Nutrition*
Mardi Mountford, *IFC*
Norriel Nipales, *Wyeth Nutrition (formerly Pfizer)*
Lawrence Pacquette, *Abbott Nutrition*
Shang-Jing Pan, *Abbott Nutrition*
Eric Poitevin, *Nestlé Research Center*
Al Poland, *AOAC Consultant*
Robert Rankin, *IFC*
Lars Reimann, *Eurofins*
Joe Romano, *Waters Corporation*
Steve Royce, *Agilent Technologies, Inc.*
Louis Salvati, *Abbott Nutrition*
Dan Schmitz, *Abbott Nutrition*
Matthew Sliva, *Perrigo/PBM Nutritionals*
Angela Song, *Abbott Nutrition*
Karla Steele, *Mead Johnson Nutrition*
John Szpylka, *Merieux NutriSciences*
Joseph J. Thompson, *Abbott Nutrition*
Linda Thompson, *Abbott Nutrition*
Melissa Thompson, *Covance Laboratories*
Laszlo Torma, *Pickering Laboratories, Inc.*
Marina Torres Rodriguez, *LATU*
Martijn Vermeulen, *TNO*
Wayne Wargo, *Abbott Nutrition*
Guy Weerasekera, *Mead Johnson Nutrition*
Laura Wood, *NIST*
David Woollard, *Hill Laboratories*
Wayne Wolf, *USDA (Retired)*
Jinchuan Yang, *Waters Corporation*
Linda Zhao, *Abbott Nutrition*
Yang Zhou, *Eurofins*

I. WELCOME AND INTRODUCTIONS

Darryl Sullivan welcomed all participants to the ERP meeting and introduced the ERP members.

II. REVIEW OF METHODS BY EXPERT REVIEW PANEL (ERP) FOR FIRST ACTION *OFFICIAL METHOD*^{SM} STATUS

For each method, the ERP/Working Group Co-Chairs discussed methods submitted.

- **Folate – *Chair:*** Erik Konings (Nestlé)

  Fol-20: Method was withdrawn for additional information

- **Carnitine – *Co-Chairs:*** John Austad (Covance) & Günther Raffler (CLF-Eurofins)
  Choline – *Co-Chair:* Sneh Bhandari (Silliker) & Nick Cellar (Abbott)
  - Instructions to study directors to collect choline data as well as carnitine

Method	Method Title	Reviewer(s)	N Vote	Comments
Folate	*Fol-20 - Analysis of Folic acid and 5-Methyltetrahydrofolate in Infant and Adult Nutritional formula using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry*	Adrienne McMahon Min Huang	Method withdrawn from consideration	◊ SPIFAN matrices did not produce internal peaks (repeat work) ◊ Concerned with interferences ◊ Confirmation ion is required ◊ Did not meet LOQ
Carnitine	*Carn-05 - Single Laboratory Validation of CARN-05: Determination of Free and Total Carnitine and Choline in Infant Formulas and Adult Nutritional Products*	John Austad Sneh Bhandari	John Austad moved & Sneh Bhandari second *Motion: move method to First Action *Official Method*^{SM} status **Yes- 14/ No-0 /Abstain-1**	◊ Collect choline data as well as carnitine ◊ Will not be considered dispute resolution method for choline ◊ Investigate choline adult powder milk
	Carn-06 - Simultaneous Determination of Carnitine and choline in Infant Formula, Adult/Pediatric Nutritional Formula, food and feed ESI LC-MS/MS	Günther Raffler John Austad	Method not recommended at this time	◊ Collect choline data as well as carnitine ◊ Recoveries needed at different levels ◊ Must use SPIFAN matrices ◊ Need SPIFAN data ◊ Resubmit for March 2015

III. REVIEW OF METHODS BY EXPERT REVIEW PANEL (ERP) FOR FINAL ACTION *OFFICIAL METHOD*^{SM} STATUS

The Expert Review Panel (ERP) reviewed seven (7) methods for Final Action *Official Methods*^{SM} status. Six (6) were recommended for Final Action to the Official Methods Board (OMB).

Method	Method Title	Reviewer(s)	Vote	Comments
Vitamin B_{12}	*2011.10 - Vitamin B_{12}: - Determination of Vitamin B12 in Infant Formula and Adult Nutritionals by HPLC*	John DeVries Shay Phillips	John DeVries moved & Scott Christiansen second *Motion: move to Final Action Yes- 11/ No-1 /Abstain-3 ——————— *Second vote: Yes- 11/ No-1 /Abstain-3	◊ Question about SPE overload – How to qualify ◊ Include safety for cyanide ◊ Chromatography resolution
Vitamin D	*2011.11 - Vitamin D - Determination of Vitamin D2 and D3 in Infant and Adult/Pediatric Nutritionals and Utilizing Ultra High Performance Liquid Chromatography/Tandem Mass Spectrometry (UHPLC-MS/MS)*	Sneh Bhandari Brendon Gill/ Harvey Indyk	Sneh Bhandari moved & Sarwar Gilani second *Motion: move to Final Action ——————— ~Motion: withdrawn	◊ Editorial changes ◊ Method not a significant improvement over existing method(s) ◊ 50% of samples do not apply to SMPR ◊ Qualifier removed ◊ Single platform – Blinded
Inositol	*2011.18 - Inositol - Determination of Myo-Inositol (Free and Bound as Phosphatidylinositol) in Infant Formula and Adult Nutritionals by Liquid Chromatography/Pulsed Amperometry with Column Switching*	Brendon Gill/ Harvey Indyk	Esther Campos-Gimenez moved & Melissa Phillips second *Motion: move to Final Action Yes- 12/ No-1 /Abstain-2 ——————— *Second vote: Yes- 12/ No-1 /Abstain-2	◊ Remove phosphatidylinositol (data does not support) ◊ Method does not match SMPR ◊ Sum of free myo-inositol (change total to free in method) ◊ Remove "phosphorylated forms" from applicability section ◊ Sum and Free and phosphydal Inositol; c**apture in applicability statement of SMPR.**
UTM	*2011.19 - Ultra Trace Minerals - Simultaneous Determination of Chromium, Selenium, and Molybdenum in Infant Formula and Adult/Pediatric Nutritional Formula by Inductively Coupled Plasma/Mass Spectrometry*	Min Huang Sneh Bhandari	Min Huang moved & Sneh Bhandari second *Motion: move to Final Action Yes- 13/ No-0 /Abstain-1	
Nucleotides	*2011.20 - Nucleotides - Nucleotides in Infant Formula by HPLC-UV*	Sneh Bhandari Estela Kneeteman	Sneh Bhandari moved & Estela Kneeteman second *Motion: move to Final Action Yes- 13/ No-0 /Abstain-1	

Vitamin A/E	*2012.10 - Vitamin A/E - 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 DL-α-Tocopherol Acetate) in Infant Formula and Adult Nutritionals by Normal Phase HPLC*	Scott Christiansen Jinchuan Yang	Scott Christiansen moved & Brendon Gill second *Motion: move to Final Action Yes- 13/ No-0 /Abstain-1	◊ Require use of primary standards ◊ Simplify calibration curve (all trans)
Fatty Acids	*2012.13 - Fatty Acids - Determination of Fatty Acids, including LCPUFAs, in Infant and Adult/Pediatric Nutritional Formula*	Jon DeVries Karen Schimpf	Jon DeVries moved & Karen Schimpf second *Motion: move to Final Action Yes- 13/ No-0 /Abstain-1	◊ Reference materials with higher RSD ◊ Double check chromatography for clarity (peaks)

IV. NEXT STEPS/FEEDBACK FROM ERP

Darryl Sullivan provided next steps including deadline dates. The ERP provided feedback on lessons learned from reviews.

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2011.10 Vitamin B12	• Safety concerns addressed • ERP recognizes & accepted that the reproducibility (RSDR)was higher than the SMPR requirement o For matrices with high RSDR the corresponding Horrat values were acceptable. The B12 levels in these samples were of low concentration. o RSDr were also high for these matrices o The stakeholders also accepted the higher RSDR • User comments were addressed Doug moved & Tom second to move 2011.10 to final action Vote: 1 abstention

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2011.18 Inositol	• the applicability in the SMPR can be interpreted more than one way • the method can measure total Inositol and PI (makes small contribution to the total) • the majority of the ERP accepted this interpretation • with the caveat that the method be revised to specify exactly what it measures • ERP recognizes & accepted that the reproducibility (RSDR) for one sample was higher than the SMPR requirement o For matrices with high RSDR the corresponding Horrat values were acceptable. The Inositol levels in these samples were of low concentration. • User comments were addressed by the ERP Yvonne moved and Tom second to move 2011.18 to final action Vote: 1 abstention

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2011.19 UTM	• User comments were distributed in the data review packet; no further discussion by the ERP (page 76 of the OMB meeting book (9-11-14)) o Per ERP member comment, methanol interference issue was previously discussed by the ERP • The ERP vote to move to final action was unanimous • The SMPR was met Doug moved and Erin second to move 2011.19 to final action Vote: 1 abstention Methanol interference issue was discussed with the method author. Author's opinion is the user comment is not relevant to the method.

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2011.20 Nucleotides	• Return to ERP; answer questions from user comments page 77 of the OMB meeting book (9-11-14) • OMB approves final action status on this method, upon response from the ERP that user comments were addressed Sneh moved and Erin second to move 2011.20 to final action upon response from the ERP chair that user comments were addressed Vote: Unanimous

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2012.10 Vitamin A/E	• ERP recognizes & accepted that the reproducibility (RSDR) for cis vitamin A in some samples were higher than the SMPR requirement o The level of cis vitamin A were low in corresponding samples • Return to ERP; answer questions from user comments page 80 of the OMB meeting book (9-11-14) • OMB approves final action status on this method, upon response from the ERP that user comments were addressed Brad moved and Erin second to move 2012.10 to final action upon response from the ERP chair that user comments were addressed Vote: Unanimous

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2012.13 Fatty Acids	• SMPR was not met • ERP recognizes & accepted that the reproducibility (RSDR) for trans fatty acid in some samples were higher than the SMPR requirement o The level of trans fatty acid were low in corresponding samples • Documentation by ERP that safety concerns were addressed • Modify safety concerns in OMA – refer to page 237 (warning statement) of the OMB meeting book (9-11-14) • OMB requires response from the ERP that user comments were addressed – page 82-83 of the OMB meeting book (9-11-14) Joe moved and Sneh second to move 2012.13 to final action upon response from the ERP chair that user comments are addressed & safety statements added to OMA Vote: 8-yes/2 – no (safety concerns)

*****Additional Comments:***

- Include First to Final Action document with all response
- Teleconference with OMB & ERP Chairs to discuss comments

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2011.10 Vitamin B12	• Safety concerns addressed • ERP recognizes & accepted that the reproducibility (RSDR)was higher than the SMPR requirement o For matrices with high RSDR the corresponding Horrat values were acceptable. The B12 levels in these samples were of low concentration. o RSDr were also high for these matrices o The stakeholders also accepted the higher RSDR • User comments were addressed Doug moved & Tom second to move 2011.10 to final action Vote: 1 abstention

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2011.18 Inositol	• the applicability in the SMPR can be interpreted more than one way • the method can measure total Inositol and PI (makes small contribution to the total) • the majority of the ERP accepted this interpretation • with the caveat that the method be revised to specify exactly what it measures • ERP recognizes & accepted that the reproducibility (RSDR) for one sample was higher than the SMPR requirement o For matrices with high RSDR the corresponding Horrat values were acceptable. The Inositol levels in these samples were of low concentration. • User comments were addressed by the ERP Yvonne moved and Tom second to move 2011.18 to final action Vote: 1 abstention

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2011.19 UTM	• User comments were distributed in the data review packet; no further discussion by the ERP (page 76 of the OMB meeting book (9-11-14)) o Per ERP member comment, methanol interference issue was previously discussed by the ERP • The ERP vote to move to final action was unanimous • The SMPR was met Doug moved and Erin second to move 2011.19 to final action Vote: 1 abstention Methanol interference issue was discussed with the method author. Author's opinion is the user comment is not relevant to the method.

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2011.20 Nucleotides	• Return to ERP; answer questions from user comments page 77 of the OMB meeting book (9-11-14) • OMB approves final action status on this method, upon response from the ERP that user comments were addressed Sneh moved and Erin second to move 2011.20 to final action upon response from the ERP chair that user comments were addressed Vote: Unanimous

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2012.10 Vitamin A/E	• ERP recognizes & accepted that the reproducibility (RSDR) for cis vitamin A in some samples were higher than the SMPR requirement o The level of cis vitamin A were low in corresponding samples • Return to ERP; answer questions from user comments page 80 of the OMB meeting book (9-11-14) • OMB approves final action status on this method, upon response from the ERP that user comments were addressed Brad moved and Erin second to move 2012.10 to final action upon response from the ERP chair that user comments were addressed Vote: Unanimous

AOAC INTERNATIONAL

Stakeholder Panel for Infant Formula and Adult Nutritionals (SPIFAN)

2012.13 Fatty Acids	• SMPR was not met • ERP recognizes & accepted that the reproducibility (RSDR) for trans fatty acid in some samples were higher than the SMPR requirement o The level of trans fatty acid were low in corresponding samples • Documentation by ERP that safety concerns were addressed • Modify safety concerns in OMA – refer to page 237 (warning statement) of the OMB meeting book (9-11-14) • OMB requires response from the ERP that user comments were addressed – page 82-83 of the OMB meeting book (9-11-14) Joe moved and Sneh second to move 2012.13 to final action upon response from the ERP chair that user comments are addressed & safety statements added to OMA Vote: 8-yes/2 – no (safety concerns)

*****Additional Comments:***

- Include First to Final Action document with all response
- Teleconference with OMB & ERP Chairs to discuss comments

MEMORANDUM

DATE: October 13, 2014

TO: AOAC® OFFICIAL METHODS BOARD MEMBERS

FROM: DARRYL SULLIVAN, CHAIR – AOAC ERP ON SPIFAN NUTRIENT METHODS

SUBJECT: Final Action Method Recommendations – Responses to OMB

AOAC OMA #	OMB Comments	ERP Chair Responses
2011.20 Nucleotides	1. Return to ERP; answer questions from user comments page 77 of the OMB meeting book (9-11-14) a. OMB approves final action status on this method, upon response from the ERP that user comments were addressed	USER COMMENTS: The ERP reviewed the comments from one laboratory regarding the method being time consuming and not complete. The ERP agreed that this comment did not reflect the overall quality of the study.
2012.10 Vitamins A & E	1. ERP recognizes & accepted that the reproducibility (RSDR) for cis vitamin A in some samples were higher than the SMPR requirement a. The level of cis vitamin A were low in corresponding samples 2. Return to ERP; answer questions from user comments page 80 of the OMB meeting book (9-11-14) a. OMB approves final action status on this method, upon response from the ERP that user comments were addressed	USER COMMENTS: The ERP reviewed the comments from one laboratory suggesting that the chromatography required improvement. All of the other participating labs found the chromatography to be acceptable. The ERP agreed that this comment did not reflect the true quality of the study.
2012.13 Fatty Acids	1. SMPR was not met 2. ERP recognizes & accepted that the reproducibility (RSDR) for trans fatty acid in some samples were higher than the SMPR requirement 3. The level of trans fatty acid were low in corresponding samples 4. Documentation by ERP that safety concerns were addressed 5. Modify safety concerns in OMA – refer to	USER COMMENTS: The ERP reviewed the user comments regarding simplifying the sample preparation procedure and the addition of more standards. Since the sample preparation and standards were acceptable to all other laboratories, the ERP agreed that these comments did not reflect the overall quality of the study.

	page 237 (warning statement) of the OMB meeting book (9-11-14) 6. OMB requires response from the ERP that user comments were addressed – page 82-83 of the OMB meeting book (9-11-14) a. OMB approves final action status on this method, upon response from the ERP that user comments were addressed & safety statements added to OMA	SAFETY STATEMENTS: During the review of AOAC 2012.13 Fatty Acids in Infant Formula and Adult Nutritionals there was concern regarding the Safety information that was in the ISO version, but not in the OMA. After consultation with Bob Rathbone of Publications, it appears that the section was omitted in error. The safety section was not omitted by the ERP, but rather as the method was rewritten into OMA format. Since this is an editorial issue, the section will be added to the OMA. I hope this provides clarity on that issue. Please let me know if you have further questions. Thank you much.

AOAC AWARDS- **2016** METHOD OF THE YEAR (ERP Chair Nomination Form)

Submission Date	2016-04-25 16:11:33
Name	Darryl Sullivan
Panel Name	SPIFAN
E-mail	darryl.sullivan@covance.com
Date	04-25-2016
AOAC Method Number	2011.10
Method Name	Determination of Vitamin B12 in Infant, Adult, and Pediatric Formulas by HPLC-UV and Column Switching
Rationale (based on selection criteria)	This is one of the most successful methods in the history of AOAC. It is not only approved as an AOAC Final Action Method, but it has also been approved by ISO, IDF, and Codex - as a Type II dispute resolution method. The SLV and MLV studies were both done extremely well and the data are amazing. The method will have a huge impact on the infant formula community, because it replaces a 50 year old microbiological method.
AOAC Method Number	2011.20
Method Name	Analysis of Nucleotide 5'-Mono phosphates in Infant Formulas by HPLC-UV
Rationale (based on selection criteria)	This is one of the most successful methods in the history of AOAC. It is not only approved as an AOAC Final Action Method, but it has also been approved by ISO, IDF, and Codex - as a Type II dispute resolution method. The SLV and MLV studies were both done extremely well and the data are amazing. The method will have a huge impact on the infant formula community, because it provides the first official method for measuring nucleotides - an extremely important nutrient that is added to almost all infant formula.
AOAC Method Number	2012.15
Method Name	Determination of Iodine in Infant Formula and Adult / Pediatric Nutritional Formula by Inductively Couple Plasma-Mass Spectrometry (ICP-MS)
Rationale (based on selection criteria)	This is one of the most successful methods in the history of AOAC. It is not only approved as an AOAC Final Action Method, but it has also been approved by ISO, IDF, and Codex - as a Type II dispute resolution method. The SLV and MLV studies were both done extremely well and the data are amazing. The method will have a huge impact on the infant formula community, because it replaces a 50 year ion specific electrode method that was prone to false positives and was extremely problematic. This is the first ICP-MS method to gain official method status for the determination of nutrients - and has set the framework for many more in the future.

AOAC AWARDS- **2016** METHOD OF THE YEAR (ERP Chair Nomination Form)

Submission Date	2016-04-28 10:57:44
Name	Wendy McMahon
Panel Name	Microbiology Methods for Food and Environmental Surfaces
E-mail	wendy.mcmahon@mxns.com
Date	04-28-2016
AOAC Method Number	2014.05
Method Name	Rapid Yeast And Mold
Rationale (based on selection criteria)	Rapid Yeast and Mold Petrifilm (RYM) by 3M was approved for First Action in December 2014 (OMA 2014.05). RYM is a practical solution to a challenging problem for many laboratories. It offers a shorter time to result which is closer to other analyses that may be performed (i.e. APC, Salmonella) allowing for companies to release product to the marketplace sooner. The test offers yeast and mold results as early as 48 hours. Another noteworthy aspect of the method is the improvement in reading colonies compared to their traditional method. 3M made this improvement by reducing spreading of molds that provides clearer colony formation and easier, more accurate counting of CFUs. Upon review of 3M's collaborative study, the recommendation of the Microbiology ERP to move the method to first action was unanimous.