INFANT FORMULA AND ADULT NUTRITIONALS

Determination of Vitamin E and Vitamin A in Infant Formula and Adult Nutritionals by Normal-Phase High-Performance Liquid Chromatography: Collaborative Study, Final Action 2012.10

Adrienne McMahon
Wyeth Nutrition Ireland, Askeaton, Co. Limerick, Ireland

Collaborators: S. Christiansen; F-F. Chee; A. Chua; H. Braddock; B. Gill; R. De Guzman; K. Kohler; D.P. Labitan; G. Larkin; J. Le Grandois; E. Marceau; F. Martin; S. Meng Jensen; L. Meyer; D. Piot; L. Rael; K. Schimpf; M. Thompson; M. Torres

The main objective of the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) project is to establish international consensus methods for infant formula and adult nutritionals, which will benefit intermarket supply and dispute resolution. A collaborative study was conducted on AOAC First Action Method 2012.10 *Simultaneous Determination of 13-*cis *and All-*trans *Vitamin A Palmitate (Retinyl Palmitate), Vitamin A Acetate (Retinyl Acetate), and Total Vitamin E (α-Tocopherol and D-α-tocopherol acetate) in Infant Formula and Adult Nutritionals by Normal-Phase HPLC*. Fifteen laboratories from 11 countries participated in an interlaboratory study to determine 13-*cis* and all-*trans* vitamin A palmitate (retinyl palmitate), vitamin A acetate (retinyl acetate), and total vitamin E (α-tocopherol and D-α-tocopherol acetate) in infant formula and adult nutritionals by normal-phase HPLC and all laboratories returned valid data. Eighteen test portions of nine blind duplicates of a variety of infant formula and adult nutritional products were used in the study. The matrixes included milk-based and soy-based hydrolyzed protein as well as a low fat product. Each of the samples was prepared fresh and analyzed in singlicate. As the number of samples exceeded the recommended number to be prepared in a single day, analysis took place over 2 days running 12 samples on day one and 10 samples on day two. The reference standard stock was prepared once and the six-point curve diluted freshly on each day. Results obtained from all 15 laboratories are reported. The RSD_R for total vitamin A (palmitate or acetate) ranged from 6.51 to 22.61% and HorRat values ranged from 0.33 to 1.25. The RSD_R for total vitamin E (as tocopherol equivalents) ranged from 3.84 to 10.78% and HorRat values ranged from 0.27 to 1.04. Except for an adult low fat matrix which generated reproducibility RSD >40% for some isomers, most SPIFAN matrixes gave results within the acceptance criteria of <16% RSD as stated in the respective *Standard Method Performance Requirements.*

Vitamin A (retinol) is an essential nutrient for normal vision, and teeth and bone formation. An inadequate intake of vitamin A causes xerophthalmia, resulting in blindness, stunted growth, and possible death. An overdose of vitamin A is damaging to infants and adults. Vitamin A can exist in several isomeric forms and as esters. Retinyl palmitate will isomerize under thermal and photochemical stress to a variety of *cis*-isomers, of which 13-*cis* is the most common and most active (75% of *trans*). Other isomers have reduced vitamin A activity. In this method, no distinction is made between the bioactivities of the isomers; instead, all are summed against the *trans* isomer to give the total vitamin A concentration.

Although vitamin E has been known since the 1920s, its functions have only recently been defined. The principle role of vitamin E is as an antioxidant, protecting many other biochemicals from damage by active oxygen and other free radicals. It works closely with vitamin C in this respect, particularly in cell membranes. Vitamin E has eight active forms which vary in methyl-substitution in the tocol ring (α-, β-, γ-, and δ-tocopherols) and in saturation of the side chain (α-, β-, γ-, and δ-tocotrienols). In food science, only α-tocopherol is usually considered, because it is the most active and most abundant vitamer.

At the AOAC INTERNATIONAL Annual Meeting on September 29, 2012, the AOAC Expert Review Panel (ERP) for the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) Nutrient methods reviewed this method separately for vitamins A and E, including all available method validation data. Following the evaluation of the data for both methods, the ERP granted First Action status to both methods and recommended that a single method be published for the simultaneous determination of vitamin A palmitate, vitamin A acetate and total vitamin E (D-α-tocopherol and D-α-tocopherol acetate) in infant formula and adult nutritionals by normal-phase HPLC. Following the completion of a single-laboratory validation

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The method was approved by the AOAC Official Methods Board as Final Action. *See* "Standards News," (2014) *Inside Laboratory Management*, November/December issue.
The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) invites method users to provide feedback on the Final Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author.
Corresponding author's e-mail: Adrienne.McMahon@wyethnutrition.com
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(SLV) using SPIFAN matrixes (a selection of commercially available infant and adult nutritional formulas), AOAC First Action Method **2012.10** was published in the *Journal of AOAC INTERNATIONAL* in 2013 (1). Following the successful outcome of SLV, the method was chosen to go forward for multilaboratory testing (MLT; collaborative study) by the ERP at the annual meeting of AOAC INTERNATIONAL in August 2013.

MLT Protocol

As per the MLT protocol (2), the main objective of each participating laboratory was as follows:

- To run the AOAC First Action Method **2012.10** as per the described procedure.
- To perform applicable system suitability tests.
- To analyze 18 selected SPIFAN samples over 2 days in singlicate.
- To send completed data tables for the calibration standards and results, chromatograms, observations, and comments to the Study Director.

Fifteen laboratories representing commercial, industrial, and governmental laboratories in 11 countries agreed to participate in this collaborative study, which was conducted using a blind duplicate design. Each laboratory was requested to first assay two practice samples to ensure that the laboratory analyst understood the entire procedure before proceeding with testing the study samples. All laboratories were asked to provide details including calculations determining the purity of all four standards used and to provide a full set of data, including system suitability checks and chromatograms, to aid the Study Director in evaluating the results and troubleshooting if necessary. Laboratories providing inadequate initial data for the practice samples were provided with troubleshooting assistance before repeating the practice samples. Laboratories providing satisfactory data on the practice samples received a shipment of the collaborative study samples. This test set contained blind duplicates, and each laboratory analyzed each test in singlicate and reported only single-test results.

List of collaborating laboratories:

(*1*) AsureQuality Ltd, Auckland, New Zealand
(*2*) Covance Laboratories, Inc.
(*3*) Perrigo Nutritionals
(*4*) Abbott Laboratories
(*5*) Premium Laboratories, Spain
(*6*) Aerial, France
(*7*) Canadian Food Inspection Agency
(*8*) Nestlé Research Centre, Lausanne, Switzerland
(*9*) Departamento de Desarrollo de Métodos, Technological Laboratory of Uruguay (LATU), Montevideo, Uruguay
(*10*) Eurofins Steins Laboratorium A-S, Denmark
(*11*) Fonterra, New Zealand
(*12*) Mead Johnson Nutrition, Philippines
(*13*) Wyeth Nutrition Ireland
(*14*) Wyeth Nutrition Singapore
(*15*) Wyeth Nutrition Philippines

Method

A slightly modified version of AOAC First Action Method **2012.10** was followed. The main method update was the weighing of the sample diluent and sample aliquot rather than measuring volumetrically. The method, with both variable UV and fluorescence detection, allows for the simultaneous determination of vitamin A palmitate, vitamin A acetate, and total vitamin E in infant, pediatric, and adult nutritional formulas. The procedure utilizes the proteolytic enzyme papain to hydrolyze the hydrophillic protein coating of fat miscelles in milk- or soy-based formulations in an aqueous solution. The hydrophobic contents of the miscelles are then extracted quantitatively into iso-octane in a single extraction and chromatographed by normal-phase HPLC using a Zorbax (Agilent Technologies, Santa Clara, CA) NH_2 analytical column. The analytes are eluted with a gradient and D-α-tocopherol and D-α-tocopherol acetate are quantified using fluorescence detection, excitation/emission, 280/310 nm. Vitamin A palmitate (*cis* and *trans*) and vitamin A acetate (*cis* and *trans*) are quantified using UV detection at 325 nm.

This method meets the applicability statements of the in AOAC *Standard Method Performance Requirements* (SMPR®; 3) **2011.003** (4) for vitamin A and AOAC SMPR **2011.010** (5) for vitamin E as follows:

Vitamin A.—Determination of vitamin A in all forms of infant and adult or pediatric formula [powders, ready-to-feed (RTF) liquids and liquid concentrates]. For the purpose of this SMPR, vitamin A is defined as 13-*cis* and all-*trans* retinol (CAS 68-26-8), retinyl esters [retinyl palmitate (CAS 79-81-2) and retinyl acetate (CAS 127-47-9)].

Vitamin E.—Determination of vitamin E in all forms of infant and adult or pediatric formula (powders, RTF liquids and liquid concentrates), with a focus on D-α-tocopherol (CAS 59-02-9) and all-racemic α-tocopherol (CAS 1406-18-4) and their esters. Methods must be able to report the quantity of α-tocopherol and esters separately.

AOAC Official Method 2012.10
Determination of Vitamins E and A in Infant Formula and Adult Nutritionals

Normal-Phase High-Performance Liquid Chromatography
First Action 2012
Final Action 2014

ISO–AOAC Method

[Applicable to the concurrent quantitative analysis of vitamin E (α-tocopherol and α-tocopherol acetate), vitamin A palmitate, and vitamin A acetate (*cis*- and *trans*-isomers) present in milk- and soy-based infant formula and adult nutritionals and formulas containing hydrolyzed protein. Vitamin A is defined as 13-*cis* and all-*trans* retinol (CAS 68-26-8), retinyl esters [retinyl palmitate (CAS 79-81-2) and retinyl acetate (CAS 127-47-9)]. The determination of vitamin E focuses on α-tocopherol (CAS No. 59-02-9), *all*-racemic α-tocopherol (CAS No. 1406-18-4), and their esters. α-Tocopherol and esters can be reported separately.]

Caution: Correct personal and environmental safety standards shall be used while performing this analytical method. Laboratory personnel handling solvents, acids, and reagents should be knowledgeable of their potential hazards. Consult the Material Safety Data Sheets (MSDS) for information on the hazards and take proper precautions. Transfer solvents and acids inside efficient fume hoods and extractors. Ensure all glassware is free from chipping and hairline cracks.

See Tables **2012.10A** and **B** for results of the method performance studies supporting acceptance of the method.

Table 2012.10A. Method performance requirements: Single-laboratory validation (SLV) and multilaboratory testing (MLT) results summary—Vitamin A[a]

Parameter	Method performance requirements		Retinyl palmitate	Retinyl acetate
Analytical range	7.0–382.6[b]		2–450	2–450
Limit of detection (LOD)	≤2.0[b]		0.099	0.85
Limit of quantitation (LOQ)	≤7.0[b]		0.33	2.83
Repeatability (RSD_r) (SLV)	7–300[b]	≤8%	≤4.03%	≤6.56%
Intermediate precision (RSD_r) (SLV)	7–300[b]	—	≤6.23%	≤10.63%
Recovery (SLV)	90–110% (mean spiked recovery over the range of the assay)		99.13%	96.53%
Reproducibility (RSD_R) (MLT)	10–383[b]	≤16%	6.51–16.25%	11.73–22.61%

[a] Concentrations apply to (*1*) 'ready-to-feed' liquids; (*2*) reconstituted powders (25 g into 200 g water); and (*3*) liquid concentrate diluted 1:1 by weight.

[b] µg/100 g reported separately as *cis*-13 retinol and all-*trans* retinol in reconstituted final product.

A. Principle

This procedure utilizes the proteolytic enzyme papain to hydrolyze the hydrophilic protein coating of fat micelles in milk- or soy-based infant formulations in an aqueous solution. The hydrophobic contents of the micelles are then extracted quantitatively into iso-octane in a single extraction and chromatographed by normal-phase HPLC using a Zorbax® NH2 analytical column. The analytes are eluted with a gradient and α-tocopherol and α-tocopherol acetate quantified using fluorescence detection, excitation/emission, 280/310 nm. Vitamin A palmitate (*cis* and *trans*) and vitamin A acetate (*cis* and *trans*) are quantified using UV detection at 325 nm.

B. Apparatus

Common laboratory glassware and equipment and, in particular, the following:

(**a**) *HPLC system.*—Consisting of pump, autosampler, programmable UV detector operating at 325 nm for vitamin A, and a fluorescence detector (FLD) at an excitation wavelength of 280 nm and an emission wavelength of 310 nm for vitamin E.

(**b**) *HPLC column*.—Analytical normal-phase column, e.g., Zorbax NH2, 5 µm, 150 × 4.6 mm, or equivalent.

(**c**) *Water bath*.—Set at 37 ± 2°C.

(**d**) *Centrifuge*.—With adapters for 50 mL centrifuge tubes, capable of 4000 min^{-1}.

(**e**) *UV-Vis spectrophotometer*.—With 1 cm quartz cells.

(**f**) *Analytical balance*.—Weighing to four decimal places.

(**g**) *Amber HPLC vials*.—2 mL, with plastic caps and polytetrafluoroethylene (PTFE) seals.

(**h**) *Disposable centrifuge tubes*.—50 mL, e.g., Falcon (Fisher, Pittsburgh, PA), or equivalent.

(**i**) *Laboratory mechanical test tube shaker*.

(**j**) *Sonic bath*.

(**k**) *One-mark volumetric flasks*.—50 and 100 mL.

(**l**) *Vacuum filtration apparatus*.—With 0.45 µm nylon membrane.

(**m**) *Laboratory glass bottles*.—250 mL and 1 and 2 L, e.g., Duran (Wertheim/Main, Germany), or equivalent.

(**n**) *Pipettors and tips.*—Gilson P10002, or equivalent.

C. Standards

(**a**) *Vitamin A palmitate reference standard.*—Primary standard, U.S. Pharmacopeial Convention (USP; Rockville, MD, USA), or equivalent. The standard shall contain antioxidant.

Table 2012.10B. Method performance requirements: Single-laboratory validation (SLV) and multilaboratory testing (MLT) results summary—Vitamin E[a]

Parameter	Method performance requirements		α-Tocopherol	α-Tocopherol acetate
Analytical range	0.2–8[b]		0.03–8	0.02–9.4
Limit of detection (LOD)	≤0.1[b]		0.01	0.023
Limit of quantitation (LOQ)	≤0.2[b]		0.035	0.075
Repeatability (RSD_r) (SLV)	0.5–2.0[b]	≤8%	≤4.25%	≤4.39
	4–8[b]	≤6%	≤3.78%	≤3.53%
Intermediate precision (RSD_r) (SLV)	<2.0[b]	—	≤17.31%	≤10.54%
	>2.0[b]	—	≤9.24%	≤8.25%
Recovery (SLV)	90–110% (mean spiked recovery over the range of the assay)		100.60%	102.92%
Reproducibility (RSD_R) (MLT)	0.5–1.0[b]	≤22%	3.84–43.56%	—
	1.0–8.0[b]	≤16%	—	4.15–11.25%
Reproducibility (RSD_R) (MLT) total vitamin E	0.5–1.0[b]	≤22%	3.84–10.78%	
	1.0–8.0[b]	≤16%	≤12.47%	

[a] Concentrations apply to (*1*) 'ready-to-feed' liquids; (*2*) reconstituted powders (25 g into 200 g water); and (*3*) liquid concentrate diluted 1:1 by weight.

[b] mg/100 g α-tocopherol and α-tocopheryl acetate in reconstituted final product.

(**b**) *Vitamin A acetate reference standard.*—Primary standard, USP, or equivalent.

(**c**) *α-Tocopherol acetate reference standard.*—Primary standard, USP, or equivalent.

(**d**) *α-Tocopherol reference standard.*—Primary standard, USP, or equivalent.

D. Chemicals and Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

(**a**) *Methyl-t-butyl ether (also known as tert-butyl methyl ether)*.—HPLC grade.

(**b**) *n-Hexane*.—HPLC grade.

(**c**) *Ethanol*.—HPLC grade.

(**d**) *Methanol*.—HPLC grade.

(**e**) *Iso-octane (2,2,4- trimethylpentane)*.—HPLC grade.

(**f**) *Papain (from Carica papaya)*.—>3 U/mg, Sigma 76220, or equivalent.

(**g**) *Hydroquinone*.—Sigma H90031, or equivalent.

(**h**) *Glacial acetic acid*.—Analytical reagent grade.

(**i**) *Anhydrous sodium acetate*.

(**j**) *Hydrochloric acid*.—36%.

E. Solutions

(**a**) *Dilute hydrochloric acid solution*.—Dilute 100 mL of a hydrochloric acid solution with a mass fraction of 36% to 200 mL with water.

(**b**) *Papain solution, mass concentration ρ = 20 g/L*.—Dissolve 100 mg hydroquinone and 4 g anhydrous sodium acetate in approximately 80 mL water in a 100 mL one-mark volumetric flask. Adjust the pH to 5.0 with dilute hydrochloric acid solution. Add 2 g papain and make up to volume. Prepare fresh prior to use.

(**c**) *Acidified methanol solution*.—Add 20 mL glacial acetic acid to 1 L methanol and mix. Prepare fresh on day of use.

(**d**) *HPLC mobile phase A*.—*n*-Hexane, filtered and degassed for 15 min in an ultrasonic bath.

(**e**) *HPLC mobile phase B*.—Mix 750 mL *n*-hexane with 250 mL methyl-*t*-butyl ether. Add 3 mL methanol, filter, and degas for 15 min in an ultrasonic bath.

F. Calibration Standards

(**a**) *Retinyl palmitate stock standard solution*.—Weigh to the nearest 0.01 mg approximately 70 mg retinyl palmitate into a 50 mL volumetric flask. Dissolve in and dilute to volume with iso-octane.

Table 2012.10C. Pump gradient elution cycle

Time, min	Flow, mL/min	Mobile phase A, %	Mobile phase B, %
0.0	1.5	95	5
3.0	1.5	95	5
12.0	1.5	5	95
14.0	1.5	5	95
15.0	1.5	95	5
20.0	1.5	95	5

(**b**) *Retinyl acetate stock standard solution*.—Weigh to the nearest 0.01 mg approximately 35 mg retinyl acetate into a 50 mL volumetric flask. Dissolve in and dilute to volume with ethanol.

(**c**) *α-Tocopherol acetate stock standard solution*.—Weigh to the nearest 0.01 mg approximately 180 mg α-tocopherol acetate into a 50 mL volumetric flask. Dissolve in and dilute to volume with iso-octane.

(**d**) *α-Tocopherol stock standard solution*.—Weigh to the nearest 0.01 mg approximately 100 mg α-tocopherol into a 50 mL volumetric flask. Dissolve in and dilute to volume with iso-octane.

Note: The above stock standard solutions are stable in a refrigerator at 4–8°C for up to 7 days.

(**e**) *Combined working standard solution 1*.—Transfer by pipet 4 mL retinyl palmitate stock standard solution, 4 mL retinyl acetate stock standard solution, 7 mL α-tocopherol acetate stock standard solution, and 20 mL α-tocopherol stock standard solution into a 50 mL volumetric flask and dilute to volume with iso-octane. Prepare this solution freshly prior to use.

(**f**) *Combined working standard solution 2*.—Transfer by pipet 8 mL combined working standard solution 1 into a 100 mL volumetric flask and dilute to volume with iso-octane. Prepare this solution freshly prior to use.

(**g**) *Calibration standard solutions*.—Into separate 50 mL volumetric flasks, transfer by pipet 0.5, 2, 4, 8, 16, and 32 mL combined working standard solution 2, and dilute to volume with iso-octane. These solutions are used to construct a multipoint calibration curve. Prepare these solutions daily prior to use.

Note: For routine testing and depending on the concentration range of the analytes in the test samples, a 3- or 4-point standard curve can be used, provided the ranges are within the lowest and highest points of the 6-point curve listed above.

If the result of any analyte is outside the calibration range, standard weights and/or dilutions can be adjusted accordingly.

G. Stock Standard Purity Determinations

(**a**) *Spectrometric purity of retinyl palmitate stock solution*.—(*1*) Pipet 1 mL retinyl palmitate stock standard solution into a l00 mL volumetric flask and make up to volume with ethanol.

(*2*) Determine the absorption at 325 nm, zeroed against ethanol in a 1 cm quartz cell. Repeat the reading twice, rinsing the sample cuvet with the solution before each reading.

(*3*) Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_{AP}, of retinyl palmitate using Equation 1:

$$SP_{AP} = \frac{A}{975} \times \frac{50}{m_{st}} \times \frac{100}{1} \times 10 \qquad (1)$$

where A = average absorbance reading, determined above; 975 = extinction coefficient of retinyl palmitate at 325 nm; and m_{st} = mass of the reference standard in mg.

(**b**) *Spectrometric purity of retinyl acetate stock solution*.—(*1*) Pipet 1 mL retinyl acetate stock standard solution into a l00 mL volumetric flask and make up to volume with ethanol.

(*2*) Determine the absorption at 325 nm, zeroed against ethanol, in a 1 cm quartz cell. Repeat the reading twice, rinsing the sample cuvet with the solution before each reading.

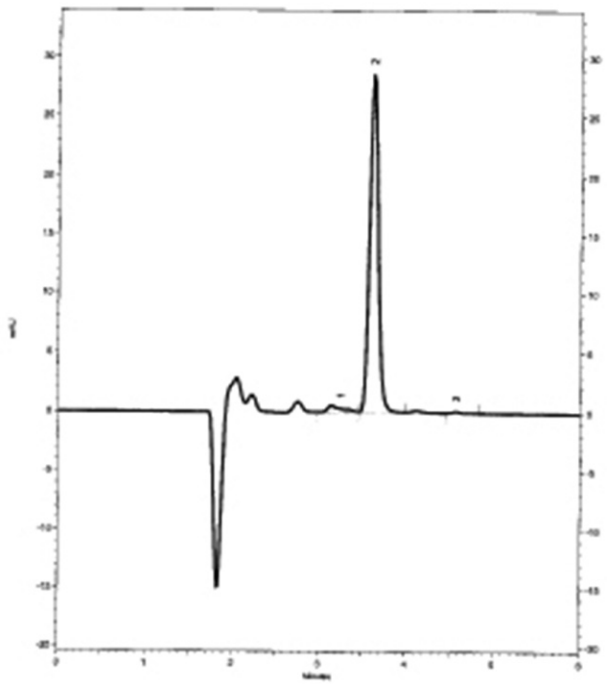

Figure 2012.10A. HPLC chromatogram of vitamin A palmitate calibration standard. Peak 1, ^{13}*cis*-isomer; peak 2, *trans*-isomer.

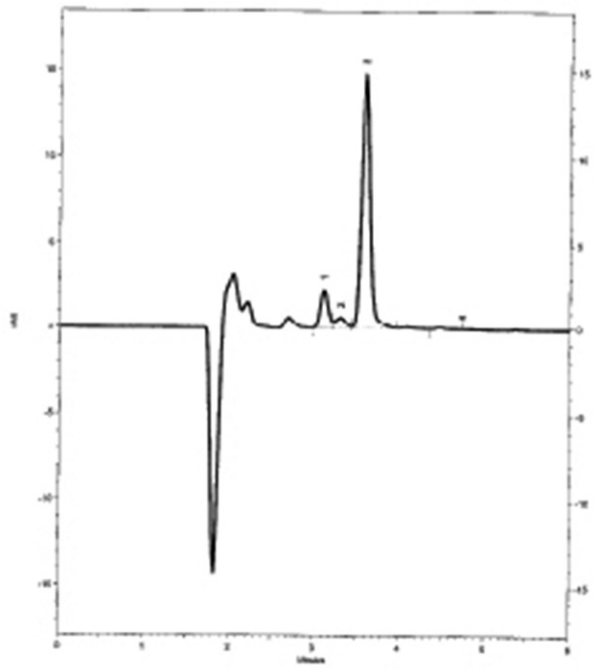

Figure 2012.10B. HPLC chromatogram of vitamin A palmitate test sample. Peak 1, ^{13}*cis*-isomer; peak 2, *cis*-isomer; peak 3, *trans*-isomer.

(*3*) Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_{AA}, of retinyl acetate using Equation 2:

$$SP_{AA} = \frac{A}{1560} \times \frac{50}{m_{st}} \times \frac{100}{1} \times 10 \qquad (2)$$

where A = average absorbance reading, determined above; 1560 = extinction coefficient of retinyl acetate at 325 nm; and m_{st} = mass of the reference standard in mg.

(**c**) *Spectrometric purity of α-tocopherol acetate stock solution.*—(*1*) Pipet 3 mL α-tocopherol acetate stock standard solution into a l00 mL volumetric flask and make up to volume with ethanol.

(*2*) Determine the absorption at 284 nm, zeroed against ethanol, in a 1 cm quartz cell. Repeat the reading twice, rinsing the sample cuvet with the solution before each reading. Calculate the average absorbance reading.

(*3*) Calculate the spectrometric purity as a decimal, SP_{TA}, of α-tocopherol acetate using Equation 3:

$$SP_{TA} = \frac{A}{43.6} \times \frac{50}{m_{st}} \times \frac{100}{3} \times 10 \qquad (3)$$

where A = average absorbance reading, determined above; 43.6 = extinction coefficient of tocopherol acetate at 284 nm; and m_{st} = mass of the reference standard in mg.

(**d**) *Spectrometric purity of α-tocopherol stock solution.*—(*1*) Pipet 3 mL α-tocopherol stock standard solution into a l00 mL volumetric flask and make up to volume with ethanol.

(*2*) Determine the absorption at 292 nm, zeroed against ethanol in a 1 cm quartz cell. Repeat the reading twice, rinsing the sample cuvet with the solution before each reading.

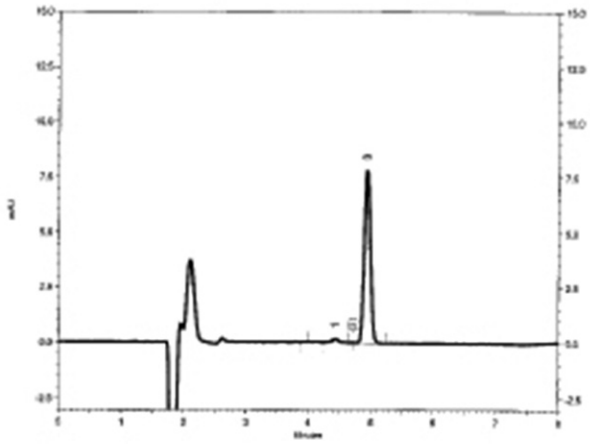

Figure 2012.10C. HPLC chromatogram of vitamin A acetate calibration standard. Peak 1, ^{13}*cis*-isomer; peak 3, *trans*-isomer.

(*3*) Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_T, of α-tocopherol using Equation 4:

$$SP_T = \frac{A}{75.8} \times \frac{50}{m_{st}} \times \frac{100}{3} \times 10 \qquad (4)$$

where A = average absorbance reading, determined above; 75.8 = extinction coefficient of tocopherol at 292 nm; and m_{st} = mass of the reference standard in mg.

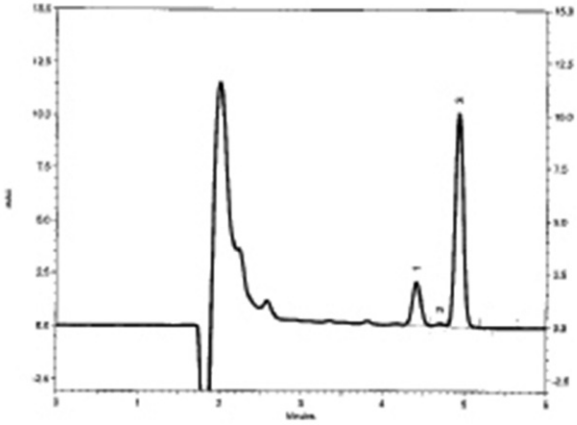

Figure 2012.10D. HPLC chromatogram of vitamin A acetate test sample. Peak 1, ^{13}*cis*-isomer; peak 2, *cis*-isomer; peak 3, *trans*-isomer.

(**e**) *Chromatographic purity of stock standard solutions*.—Prepare each stock standard solution separately as follows:

(*1*) Into four separate 100 mL volumetric flasks transfer by pipet 1 mL of each of the stock standard solutions, retinyl palmitate, retinyl acetate, α-tocopherol acetate, and α-tocopherol. Label each flask with the individual analyte names.

(*2*) Mix and dilute each to volume with iso-octane.

(*3*) Into four separate labeled 2 mL autosampler vials transfer by autopipettor 60 µL retinyl palmitate solution, 30 µL retinyl acetate solution, 100 µL α-tocopherol acetate solution, and 400 µL α-tocopherol. Fill vial with iso-octane to approximately 2 mL.

(*4*) Vortex briefly and inject into the LC system according to the method parameters described in **G**. Analyze vitamin A palmitate and vitamin A acetate by UV at 325 nm. For α-tocopherol acetate, analyze by UV at 284 nm and for α-tocopherol, analyze at 292 nm.

(*5*) Calculate the chromatographic purity (CP) as a decimal for each peak of interest after integration of all the peaks appearing on each chromatogram, using Equation 5:

$$CP = A/100 \qquad (5)$$

where CP = area of peak of interest/total peak area excluding solvent.

(**f**) *Calculation of the concentrations of working standard solutions*.—Calculate the concentration, ρ_w, of each vitamin in the working standard solutions from the stock solution concentration using the appropriate dilution factor as shown in Equations 6 to 9 in µg/mL for retinyl palmitate (RP) and retinyl acetate (RA) and mg/mL for α-tocopherol (T) and α-tocopherol acetate (TA).

$$\rho_{wRP} = SP_{RP} \times CP_{RP} \times \frac{m_{st}}{50} \times \frac{4}{50} \times \frac{8}{100} \times \frac{V_a}{50} \times 1000 \qquad (6)$$

$$\rho_{WRA} = SP_{RA} \times CP_{RA} \times \frac{m_{st}}{50} \times \frac{4}{50} \times \frac{8}{100} \times \frac{V_a}{50} \times 1000 \qquad (7)$$

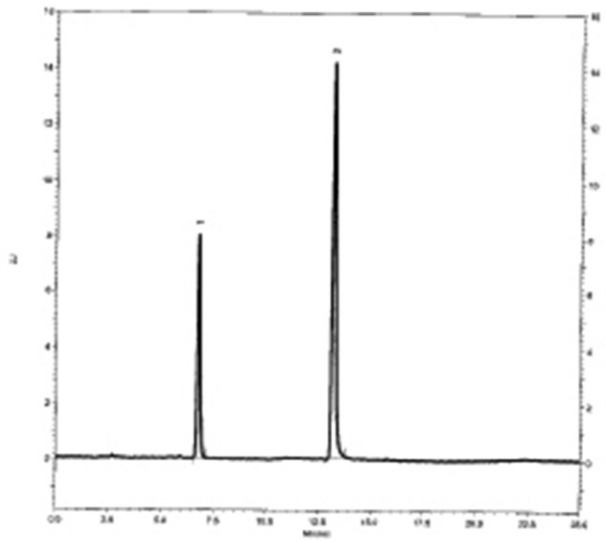

Figure 2012.10E. HPLC chromatogram of α-tocopherol acetate and α-tocopherol calibration standard. Peak 1, α-tocopherol acetate; peak 2, α-tocopherol.

$$\rho_{wT} = SP_T \times CPT \times \frac{m_{st}}{50} \times \frac{7}{50} \times \frac{8}{100} \times \frac{V_a}{50} \qquad (8)$$

$$\rho_{WTA} = SP_{TA} \times SP_{TA} \times \frac{m_{st}}{50} \times \frac{20}{50} \times \frac{8}{100} \times \frac{V_a}{50} \qquad (9)$$

where V_a = 0.5, 2, 4, 8, 16, and 32 mL, respectively, for the calibration levels; m_{st} = mass of the reference standard in mg; SP = UV spectrometric purity as a decimal; CP = chromatographic purity as a decimal; and 1000 = conversion factor from mg/mL to µg/mL.

H. Sample Preparation

(**a**) For dry blended/nonhomogenous powder samples, transfer 25 g, accurately weighed, to a 250 mL bottle. Dissolve using warm water (about 40–45°C), cool, and make up to 200 g with water. Note the final weight (m_2). Transfer 5.0 g (m_3) reconstituted sample to a screw-top centrifuge tube. Calculate the sample mass (powder equivalent), m_s, using Equation 10:

$$m_s = \frac{(m_1 \times m_3)}{m_2} \qquad (10)$$

(**b**) For wet blended homogenous powder samples, transfer 0.5000–0.5500 g, accurately weighed, directly to a screw-top 50 mL centrifuge tube. Add 5 mL warm water of approximately 40°C and shake to dissolve.

(**c**) For ready-to-feed samples or concentrated liquid products, transfer 5.0 g (m_3) thoroughly homogenized sample directly to a screw-top 50 mL centrifuge tube.

(**d**) To the above weighed solutions, add 5 mL papain solution. Mix to disperse each sample, cap, and place the tubes in a 37±2°C water bath for 20–25 min.

(**e**) Remove the samples from the water bath and cool. Place in a freezer for about 5 min or refrigerate for about 20 min.

(**f**) Add 20 mL acidified methanol to each sample tube and shake tubes for 10 min, preferably with a mechanical shaker.

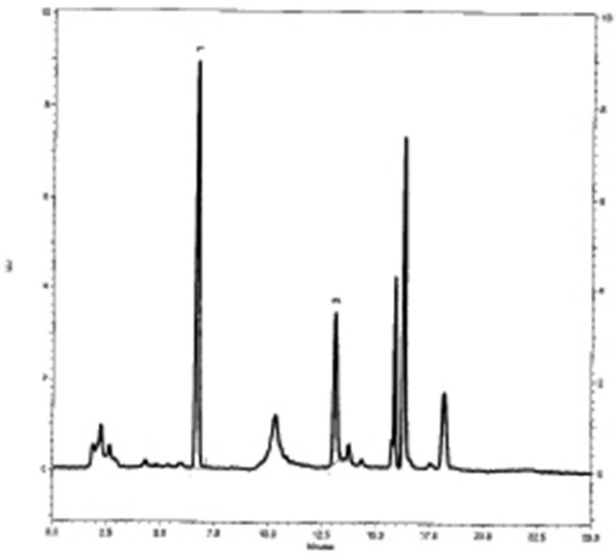

Figure 2012.10F. HPLC chromatogram of α-tocopherol acetate and α-tocopherol sample chromatogram. Peak 1, α-tocopherol acetate; peak 2, α-tocopherol.

(**g**) Volumetrically pipet 10.0 mL iso-octane to each sample tube. Close tightly to avoid leakage and shake tubes for 10 min, preferably with a mechanical shaker.

(**h**) Centrifuge for 10 min at 4000 min^{-1} to obtain a clear iso-octane layer.

(**i**) Transfer an aliquot of the clear iso-octane layer into amber vials for HPLC analysis.

I. HPLC Analysis

Separation and quantification have proven to be satisfactory if the following experimental conditions are followed:

Column.—Zorbax NH2 (5 µm, 150 × 4.6 mm).

Mobile phase A.—*n*-Hexane.

Mobile phase B.—Mixture of 750 mL *n*-hexane, 250 mL methyl-*t*-butyl ether, and 3 mL methanol.

Flow rate.—1.5 mL/min.

Injection volume.—50 µL.

Column oven.—40 ± 2°C.

Run time.—20 min.

Detector settings.—Set the photodiode array (PDA)/UV detector at 325 nm for vitamin A palmitate and vitamin A acetate. Set the fluorescence detector at excitation wavelength of 280 nm and emission wavelength of 310 nm for α-tocopherol acetate and α-tocopherol.

Pump gradient elution cycle.—*See* Table **2012.10C**.

Examples for typical chromatograms are given in Figures **2012.10A–F**.

Note: The gradient given can be altered as required to maximize the analytical separation and avoid interferences.

J. System Suitability

The following system suitability and standard checks should be met when running this method.

(**a**) The coefficient of determination, R^2, of each calibration curve should be ≥0.995.

(**b**) The resolution between *cis* and *trans* vitamin A palmitate and between *cis* and *trans* vitamin A acetate in the reference standard should be ≥1.5.

K. Calculations

Calculate the concentration, *w*, of the sample in µg/100 g for retinyl palmitate or retinyl acetate and mg/100 g for α-tocopherol or α-tocopherol acetate (powder or liquid).

$$w = \frac{(A_s - I)}{S} \times V_{iso} \times \frac{100}{m_s} \qquad (11)$$

where A = peak area or height of retinyl palmitate or retinyl acetate or α-tocopherol or α-tocopherol acetate in the test sample solution; I = intercept of the calibration curve; S = slope of the calibration curve; V_{iso} = volume of iso-octane used (here, V_{iso} = 10 mL); 100 = factor to convert in 100 g basis; and m_s = sample mass (for liquid samples) or powder equivalent in g (powder samples).

For the purposes of this method there is no differentiation of the varying contributions of cis- and transisomers to the total vitamin A palmitate/acetate activity.

For vitamin A peak integration, sum the area of the 13-cis and all trans isomers of vitamin A palmitate/acetate and calculate against the trans isomer.

To convert vitamin A results to retinol using stoichiometric calculations in accordance with Equation 12:

Vitamin A as retinol (µg/100 g) =
(retinyl palmitate in µg/100 g × 0.55)
+ (retinyl acetate in µg/100 g × 0.87)

Convert vitamin E results to α-tocopherol using stoichiometric calculations:

— 1 mg of α-tocopheryl acetate is equal to 1,10 α-tocopherol, and — 1 mg = 1,10 DL α-tocopherol (synthetic vitamin E; all racemic α-tocopherol).

Results and Discussion

System Suitability and Linearity

All system suitability checks performed during this collaborative study met the following acceptance criteria:

(a) The resolution between the *cis* and *trans* forms of vitamin A palmitate and *cis* and *trans* forms of vitamin A acetate were baseline separated.

(b) Standard injection precision was <2.0%.

(c) The coefficient of determination R^2 of all standard curves generated during the study exceeded the minimum requirement of ≥0.995.

Practice Samples

Two practice samples (both from milk based formula, one fortified with Vitamin A palmitate and one fortified with Vitamin A

Acetate and both fortified with α-tocopherol acetate) were used by the participating laboratories so that the laboratories could become familiar with the analysis procedure. The results were submitted to the Study Director for approval prior to commencing the collaborative study. Results within a range of expected levels indicated that the laboratory was capable of successfully running the analysis. The same practice samples were used as QC samples during the analysis of the study samples.

Most of the laboratories submitted practice sample results that met the acceptance criteria. A couple of laboratories submitted results just outside the acceptance limits but were accepted to participate in the study as they met the acceptance limits for at least three of the four analytes.

Milk-Based Products

For retinyl palmitate, RSD_r was calculated in a range of 1.06–4.72% and RSD_R in a range of 6.51–10.52%.

For retinyl acetate, RSD_r was 16.60% and RSD_R was 22.61%.

For α-tocopherol acetate, RSD_r was calculated in a range of 0.60–3.84% and RSD_R in a range of 4.15–8.67%.

For α-tocopherol, RSD_r was calculated in a range of 1.57–5.78% and RSD_R in a range of 5.68–12.47%.

For total vitamin E, RSD_r as tocopherol equivalents (TEs) was calculated in a range of 0.81–3.74% and RSD_R in a range of 3.84–7.17%.

Note: One milligram of α-tocopherol acetate is equal to 0.671 mg TEs. One milligram of α-tocopherol is equal to 1 mg TEs.

Low Fat Product

For total vitamin A palmitate, RSD_r was 15.78% and RSD_R was 21.73%.

For α-tocopherol acetate, RSD_r was 2.11% and RSD_R was 8.50%.

For α-tocopherol, RSD_r was 8.90% and RSD_R was 43.56%.

For total vitamin E, RSD_r as TEs was 2.71% and RSD_R was 10.78%.

The concentration of α-tocopherol was relatively low in the product: 0.14 mg/100 g ready to feed (RTF).

Hydrolyzed Protein and Elemental Products

For retinyl acetate, the partially hydrolyzed soy-based product gave an RSD_r of 2.30% and an RSD_R of 11.93%.

For α-tocopherol acetate, RSD_r was calculated as 3.65% and RSD_R was 11.25%.

For α-tocopherol, RSD_r was calculated as 1.67% and RSD_R was 11.94%.

For total vitamin E as TEs, RSD_r was calculated as 5.46% and RSD_R was 10.15%.

For retinyl palmitate, the elemental product gave an RSD_r of 15.13% and an RSD_R of 16.25%.

For α-tocopherol acetate, RSD_r was calculated as 3.38% and RSD_R was 6.66%.

For α-tocopherol, RSD_r was calculated as 15.48% and RSD_R was 17.44%.

For total vitamin E as TEs, RSD_r was calculated as 4.90% and RSD_R was 5.68%.

Soy Product

For retinyl palmitate, RSD_r was 6.84% and RSD_R was 9.66%.

For α-tocopherol acetate, RSD_r was calculated as 1.67% and RSD_R was 6.47%.

For α-tocopherol, RSD_r was calculated as 7.89% and RSD_R was 8.74%.

For total vitamin E as TEs, RSD_r was calculated as 2.07% and RSD_R was 4.22%.

For two blind duplicate samples of the milk-based hydrolyzed protein formula, where the repeatability was observed as being very high (up to 50% RSDs), repeat analysis was performed by a selection of laboratories. All initial results were confirmed. Repeatability obtained at the SLV stage (duplicates over 6 separate days) was very good for this matrix, 5.4 and 5.78% for *cis* and *trans* retinyl palmitate and 5.5 and 10.2% for α-tocopherol acetate and α-tocopherol. Results obtained for this sample were deemed invalid due to the material being expired at time of testing following an investigation and confirmation that other study directors encountered similar difficulties and following a discussion at the September 2014 ERP meeting. The results for this sample have been removed from the AOAC Final Action Method document.

AOAC INTERNATIONAL *Interlaboratory Study Workbook Revision 2.1* (6) was used to perform the statistical evaluation of the data. Outliers were detected and reviewed prior to exclusion from the data set. Where possible, detected outliers were reviewed by the participating laboratories for possible transcription or calculation errors. Across the four anaytes, 13-cis and all-trans Vitamin A, and α-tocopherol acetate and α-tocopherol, laboratories 4–8 and 10–12 had no statistical outliers. Laboratory 13 had one outlier, laboratories 1 and 15 had 2 outliers, laboratory 2 had 3 outliers, laboratory 3 had 4 outliers, laboratories 9 and 14 had 14 outliers. The statistical summary (Tables 1-20) lists the outliers and the reasons for removal (Cochran test outlier or Single Grubbs test outlier), as well as Cochran test or single Grubbs test outliers.

An invitation was sent to all participating laboratories to comment on the performance of the method in their laboratories. In general, the comments were very positive. Laboratory 3 indicated that sample preparation was easy. According to the laboratory, it is not necessary to prepare the working standard (6 point curve) fresh daily, as the reagent used to prepare the stock and the working standards is the same. Laboratory 3 also indicated that the papain solution should be sonicated to ensure it is fully dissolved. Laboratory 4 changed the dilution of the standards to be within the working range of the spectrophotometric measurements and HPLC calibration curves. It used an injection volume of 35 µL instead of 50. An injection volume between 20 and 100 µL can be used with the method, depending on sensitivity. The laboratory indicated that 35 µL presented areas (or heights) to give optimum quantification and optimization. Laboratory 5 commented that the sample preparation procedure is

easy, quick (high throughput), and very much fit/suitable in a routine testing environment. Laboratory 6 commented that the calibration curves for vitamin A palmitate, vitamin A acetate, and α-tocopherol were too large, so the sample weight was adjusted to fit the curves. It had no problems with sample preparation, finding it easy to do. It has an advantage over the saponification method as it measures the esters separately. Laboratory 7 found that the method offered very good precision in terms of repeatability and reproducibility. It also noted that the standards and reagents/solutions were easy to prepare and that the sample preparation was simple and stable. It indicated that the method only takes 3 h to prepare a minimum of 15 samples; even less time is needed if not reconstituting, and still allowing determination of retinyl palmitate, retinyl acetate, α-tocopherol acetate, and α-tocopherol. Saponification procedures cannot separate α-tocopherol acetate and α-tocopherol. Laboratory 10 found that the method was very straight-forward and encountered no issues with the protocol or samples. Laboratory 11 found that the method performed well in its laboratory for most of the SPIFAN matrixes, however, it experienced a few difficulties with the sample preparation and would prefer to use vortex mixers or stir plates to a mechanical shaker. It found that with some matrixes, the samples were difficult to mix well with their model of mechanical shaker.

Conclusions

The purpose of this standard is to provide a simple, accurate analytical method for the analysis of total vitamin A and total vitamin E in infant formula and adult nutritional products, while also meeting the applicability statements and complying with the performance acceptance criteria outlined in the SPIFAN SMPRs (4, 5).

Cis and *trans* isomers of vitamin A (palmitate and acetate) and α-tocopherol acetate and α-tocopherol can be separately quantified by UV and fluorescence detection. Compared with other methods for the analysis of these fat-soluble vitamins, this method is considered more rapid and efficient, providing good performance and ease of implementation for routine use in a QC environment.

The collaborative study included 15 laboratories. Some had experience in using this method while others had no previous experience. Low repeatability for the majority of matrix types across the laboratories indicates that relatively little experience is required to precisely and efficiently run this method. The method was applied to a variety of different infant formulas and adult nutritional product types and demonstrated acceptable reproducibility precision across the analytes.

Table 1. Precision data for vitamin A retinyl acetate (a and b) and retinyl palmitate (c–h)

Sample	Adult nutritional powder milk protein-based	Infant formula powder partially hydrolyzed soy-based	SRM 1849a[a]	Adult nutritional powder low fat	Infant formula			Infant elemental powder
					Powder soy-based	Powder milk-based	RTF milk-based[b]	
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
No. of laboratories	15	15	15	15	15	15	15	15
No. of laboratories retained after eliminating outliers	15	12	15	15	15	14	14	15
No. of outliers (laboratories)	0	3	0	0	0	1	1	0
No. of accepted results	30	24[c,d]	30	30	30	28[c]	28[c]	30
Mean value $\bar{x}$, µg/100 g RTF	46.34	67.39	6.49	47.55	62.56	66.58	57.34	48.35
Repeatability SD s_r, µg/100 g RTF	7.69	1.55	0.21	7.50	4.28	0.75	0.61	7.31
Reproducibility SD s_R, µg/100 g RTF	10.48	8.04	0.52	10.33	6.04	4.33	4.13	7.86
RSD_r, %	16.60	2.30	3.26	15.78	6.84	1.13	1.06	15.13
RSD_R, %	22.61	11.93	8.02	21.73	9.66	6.51	7.20	16.25
Repeatability limit r ($r = 2.8 \times s_r$), µg/100 g RTF	21.54	4.34	0.59	21.01	11.98	2.10	1.70	20.48
Reproducibility limit R ($R = 2.8 \times s_R$), µg/100 g RTF	29.34	22.52	1.46	28.92	16.91	12.13	11.56	21.99
HorRat value	1.25	0.69	0.33	1.20	0.56	0.38	0.41	0.91

[a] Milligrams per kilogram powder (National Institute of Standards and Technology, Gaithersburg, MD).

[b] RTF = Ready-to-feed.

[c] Cochran test outlier.

[d] Grubbs test outlier.

Table 2. MLT precision data for vitamin E D-α-tocopherol

Sample	Adult nutritional powder milk protein-based	Infant formula powder partially hydrolyzed soy-based	SRM 1849a[a]	Adult nutritional powder low fat	Infant formula			Infant elemental powder
					Powder soy-based	Powder milk-based	RTF milk-based[b]	
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
No. of laboratories	15	15	15	15	15	15	15	15
No. of laboratories retained after eliminating outliers	14	11	14	14	14	13	14	14
No. of outliers (laboratories)	1	4	1	1	1	0	1	1
No. of accepted results	28[c]	22[c,d]	28[c]	28[d]	28[c,d]	26[c,d]	28[c]	28[c]
Mean value $\bar{x}$, mg/100 g RTF	0.58	0.48	36.38	0.14	0.46	0.49	0.45	0.41
Repeatability SD s_r, mg/100 g RTF	0.023	0.008	2.103	0.012	0.036	0.008	0.012	0.064
Reproducibility SD s_R, mg/100 g RTF	0.052	0.057	4.539	0.061	0.040	0.279	0.035	0.072
RSD_r, %	3.99	1.67	5.78	8.90	7.89	1.57	2.59	15.48
RSD_R, %	9.10	11.94	12.47	43.56	8.74	5.68	7.73	17.44
Repeatability limit r ($r = 2.8 \times s_r$), mg/100 g RTF	0.064	0.022	5.889	0.035	0.101	0.022	0.033	0.178
Reproducibility limit R ($R = 2.8 \times s_R$), mg/100 g RTF	0.147	0.159	12.71	0.170	0.112	0.078	0.098	0.201
HorRat value	0.930	0.920	1.870	2.180	0.680	0.440	0.600	1.340

[a] Milligrams per kilogram powder.

[b] RTF = Ready-to-feed.

[c] Grubbs test outlier.

[d] Cochran test outlier.

Table 3. MLT precision data for vitamin E D-α-tocopherol acetate

Sample	Adult nutritional powder milk protein-based	Infant formula powder partially hydrolyzed soy-based	SRM 1849a[a]	Adult nutritional powder low fat	Infant formula			Infant elemental powder
					Powder soy-based	Powder milk-based	RTF milk-based[b]	
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
No. of laboratories	15	15	15	15	15	15	15	15
No. of laboratories retained after eliminating outliers	14	15	14	13	14	13	13	15
No. of outliers (laboratories)	1	0	0	0	0	2	2	0
No. of accepted results	28[c]	30	28[d]	26[c,d]	28[d]	26[c,d]	26[c,d]	30
Mean value $\bar{x}$, mg/100 g RTF	12.73	1.80	172.89	1.84	1.30	1.44	1.56	1.79
Repeatability SD s_r, mg/100 g RTF	0.489	0.066	3.374	0.388	0.022	0.021	0.009	0.061
Reproducibility SD s_R, mg/100 g RTF	0.926	0.203	14.991	0.156	0.084	0.067	0.065	0.119
RSD_r, %	3.84	3.65	1.95	2.11	1.67	1.43	0.60	3.38
RSD_R, %	7.28	11.25	8.67	8.50	6.47	4.62	4.15	6.66
Repeatability limit r ($r = 2.8 \times s_r$), mg/100 g RTF	1.369	0.184	9.447	0.109	0.061	0.058	0.026	0.170
Reproducibility limit R ($R = 2.8 \times s_R$), mg/100 g RTF	2.594	0.567	41.976	0.437	0.236	0.187	0.181	0.334
HorRat value	0.330	1.070	0.580	0.810	0.580	0.420	0.380	0.630

[a] Milligrams per kilogram powder.

[b] RTF = Ready-to-feed.

[c] Grubbs test outlier.

[d] Cochran test outlier.

Table 4. MLT precision data for total vitamin E as TE

Sample	Adult nutritional powder milk protein-based	Infant formula powder partially hydrolyzed soy-based	SRM 1849a[a]	Adult nutritional powder low fat	Infant formula			Infant elemental powder
					Powder soy-based	Powder milk-based	RTF milk-based[b]	
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
No. of laboratories	15	15	15	15	15	15	15	15
No. of laboratories retained after eliminating outliers	14	15	12	14	14	14	14	15
No. of outliers (laboratories)	1	0	3	1	1	1	1	0
No. of accepted results	28[c]	30	24[c,d]	28[d]	28[d]	28[d]	28[d]	30
Mean value $\bar{x}$, mg/100 g RTF	13.30	2.27	209.24	1.95	1.79	1.94	2.01	2.22
Repeatability SD s_r, mg/100 g RTF	0.50	0.12	4.06	0.05	0.04	0.03	0.02	0.10
Reproducibility SD s_R, mg/100 g RTF	0.95	0.23	8.18	0.21	0.08	0.08	0.08	0.12
RSD_r, %	3.74	5.46	1.94	2.71	2.07	1.32	0.81	4.70
RSD_R, %	7.17	10.15	3.91	10.78	4.22	4.13	3.84	5.55
Repeatability limit r ($r = 2.8 \times s_r$), mg/100 g RTF	10.46	15.28	5.44	7.58	5.79	3.70	2.27	13.17
Reproducibility limit R ($R = 2.8 \times s_R$), mg/100 g RTF	20.09	28.43	10.94	30.19	11.81	11.57	10.74	15.53
HorRat value	0.93	1.00	0.27	1.04	0.68	0.40	0.37	0.55

[a] Milligrams per kilogram powder.

[b] RTF = Ready-to-feed.

[c] Grubbs test outlier.

[d] Cochran test outlier.

Table 5. Collaborative data for adult nutritional powder milk protein-based

Laboratory No.	*Cis*		*Trans*		Total vitamin A as RE (retinol equivalents)	
	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	2.56	2.70	43.47	46.68	46.03	49.38
Laboratory 2	2.55	2.18	47.96	37.63	50.51	39.80
Laboratory 3	3.06	2.32	52.54	39.76	55.60	42.08
Laboratory 4	3.39	3.20	60.76	50.04	64.15	53.24
Laboratory 5	2.50	2.63	40.60	43.45	43.10	46.08
Laboratory 6	2.28	2.01	38.06	38.98	40.34	40.99
Laboratory 7	4.07	2.62	62.54	43.91	66.61	46.53
Laboratory 8	3.18	2.93	38.38	49.62	41.56	52.55
Laboratory 9	3.13	12.10	39.57	35.78	42.70	47.88
Laboratory 10	2.33	2.18	44.52	35.77	46.85	37.96
Laboratory 11	1.69	2.45	31.58	45.08	33.28	47.53
Laboratory 12	2.36	1.88	38.81	31.05	41.17	32.93
Laboratory 13	1.62	3.12	29.09	47.49	30.70	50.61
Laboratory 14	8.04	4.31	58.14	64.81	66.18	69.12
Laboratory 15	2.05	1.84	27.46	33.38	29.51	35.22

Table 6. Collaborative data for infant formula powder partially hydrolyzed soy-based

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	15.71	15.71	47.93	51.11	63.64	66.83
Laboratory 2	16.45[a]	6.64[a]	58.37[a]	18.22[a]	74.82[a]	24.86[a]
Laboratory 3	16.00[a]	7.83[a]	51.48[a]	20.63[a]	67.48[a]	28.45[a]
Laboratory 4	19.53	19.22	61.29	58.14	80.83	77.36
Laboratory 5	17.89	17.88	50.59	50.84	68.48	68.71
Laboratory 6	15.22	14.09	51.87	48.88	67.09	62.97
Laboratory 7	20.56	19.54	62.14	64.28	82.70	83.82
Laboratory 8	15.83	16.67	52.36	52.19	68.18	68.86
Laboratory 9	14.34	15.23	46.86	47.93	61.20	63.16
Laboratory 10	17.11	17.12	54.69	54.70	71.79	71.82
Laboratory 11	14.76	15.44	49.92	49.87	64.67	65.30
Laboratory 12	14.82	15.90	49.03	46.91	63.85	62.81
Laboratory 13	14.28	14.56	51.25	47.75	65.53	62.31
Laboratory 14	25.67[a]	27.03[a]	93.72[a]	84.35[a]	119.39[a]	111.38[a]
Laboratory 15	12.66	12.66	39.58	40.53	52.24	53.20

[a] Cochran and/or Grubbs outliers.

Table 7. Collaborative data for SRM 1849a

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	1.31	1.31	4.76	4.73	6.08	6.03
Laboratory 2	1.53	1.55	5.41	5.40	6.94	6.95
Laboratory 3	1.43	1.37	4.96	4.83	6.39	6.19
Laboratory 4	1.52	1.50	5.19	5.20	6.71	6.69
Laboratory 5	1.41	1.51	4.83	5.09	6.23	6.60
Laboratory 6	1.26	1.21	4.41	4.49	5.68	5.69
Laboratory 7	1.52	1.52	5.20	5.12	6.72	6.65
Laboratory 8	1.44	1.48	5.21	5.31	6.65	6.80
Laboratory 9	1.45	1.22	5.03	4.32	6.48	5.54
Laboratory 10	1.52	1.72	5.43	5.66	6.95	7.37
Laboratory 11	1.46	1.58	5.10	5.23	6.56	6.81
Laboratory 12	1.36	1.40	4.78	4.80	6.14	6.19
Laboratory 13	1.65	1.63	5.91	5.88	7.56	7.51
Laboratory 14	1.42	1.38	5.05	4.98	6.47	6.36
Laboratory 15	1.28	1.26	4.62	4.66	5.90	5.92

Table 8. Collaborative data for adult nutritional powder low fat

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	6.67	10.34	29.25	44.20	35.92	54.54
Laboratory 2	8.34	10.75	38.91	50.02	47.25	60.77
Laboratory 3	8.72	7.81	40.21	35.86	48.93	43.67
Laboratory 4	8.68	11.19	38.52	48.00	47.20	59.19
Laboratory 5	10.82	7.87	46.60	35.12	57.42	42.98
Laboratory 6	9.09	9.39	41.89	42.12	50.98	51.51
Laboratory 7	8.15	8.22	37.78	39.49	45.93	47.71
Laboratory 8	8.20	10.21	39.05	47.04	47.26	57.25
Laboratory 9	9.32	5.39	41.30	22.57	50.62	27.96
Laboratory 10	11.07	10.11	48.76	47.89	59.83	57.99
Laboratory 11	9.25	11.33	38.73	47.02	47.98	58.35
Laboratory 12	9.33	8.34	42.29	39.42	51.62	47.76
Laboratory 13	3.84	4.66	22.17	25.92	26.01	30.58
Laboratory 14	9.74	9.53	46.56	43.99	56.30	53.53
Laboratory 15	4.85	5.38	23.41	25.81	28.26	31.19

Table 9. Collaborative data for infant elemental powder

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	3.53	3.09	46.48	37.57	50.01	40.66
Laboratory 2	3.16	3.13	37.82	37.10	40.98	40.23
Laboratory 3	4.70	3.60	52.84	44.01	57.53	47.61
Laboratory 4	3.02	3.43	42.54	39.20	45.56	42.63
Laboratory 5	4.05	5.21	40.44	51.70	44.49	56.90
Laboratory 6	3.32	2.74	43.05	35.83	46.37	38.57
Laboratory 7	3.53	5.55	43.61	57.42	47.14	62.98
Laboratory 8	3.73	3.89	43.75	46.90	47.48	50.79
Laboratory 9	5.30	4.47	51.09	44.48	56.38	48.95
Laboratory 10	4.47	7.88	41.01	61.67	45.48	69.55
Laboratory 11	3.70	2.85	34.23	35.44	37.94	38.29
Laboratory 12	2.45	3.78	45.00	52.41	47.45	56.18
Laboratory 13	2.46	2.27	40.45	37.83	42.91	40.10
Laboratory 14	5.24	3.66	55.56	43.28	60.80	46.94
Laboratory 15	3.73	4.19	43.66	47.89	47.39	52.08

Table 10. Collaborative data for infant formula powder milk-based

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	11.94	11.92	54.49	54.06	66.43	65.98
Laboratory 2	12.49	12.52	56.41	55.72	68.90	68.23
Laboratory 3	11.67	11.68	53.73	54.04	65.40	65.72
Laboratory 4	10.96	10.86	56.69	56.33	67.65	67.19
Laboratory 5	12.93	12.92	56.22	56.48	69.15	69.40
Laboratory 6	10.19	10.37	51.70	51.13	61.89	61.50
Laboratory 7	12.76	12.78	54.63	56.40	67.39	69.18
Laboratory 8	11.70	11.81	56.37	57.88	68.07	69.69
Laboratory 9	12.12[a]	9.20[a]	52.39[a]	39.08[a]	64.50	48.27
Laboratory 10	14.56	14.29	62.41	61.46	76.96	75.75
Laboratory 11	12.29	12.30	56.16	54.91	68.45	67.21
Laboratory 12	10.60	10.58	54.36	53.18	64.96	63.75
Laboratory 13	10.05	9.73	50.44	50.59	60.49	60.32
Laboratory 14	11.96	12.49	55.00	56.53	66.96	69.02
Laboratory 15	10.43	10.21	48.83	49.11	59.26	59.31

[a] Cochran and/or Grubbs outliers.

Table 11. Collaborative data for infant formula RTF milk-based

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	16.23	16.33	37.54	36.91	53.77	53.24
Laboratory 2	18.06	17.89	42.26	42.34	60.31	60.23
Laboratory 3	17.40	17.30	40.96	40.25	58.36	57.55
Laboratory 4	17.17	17.36	40.45	40.75	57.61	58.11
Laboratory 5	18.17	17.96	41.56	41.42	59.73	59.38
Laboratory 6	14.95	15.48	37.85	37.70	52.79	53.18
Laboratory 7	18.19	18.31	40.64	41.50	58.83	59.81
Laboratory 8	17.12	17.03	41.54	41.92	58.66	58.94
Laboratory 9	16.67	14.52	38.35[a]	33.44[a]	55.02	47.96
Laboratory 10	22.42	20.57	45.97	45.55	68.39	66.12
Laboratory 11	16.90	17.12	40.63	40.69	57.53	57.81
Laboratory 12	15.61	15.82	38.11	38.26	53.73	54.08
Laboratory 13	15.29	15.52	39.92	39.66	55.21	55.18
Laboratory 14	17.72	17.16	41.13	40.39	58.85	57.55
Laboratory 15	13.80	14.25	36.05	36.49	49.85	50.74

[a] Cochran and/or Grubbs outliers.

Table 12. Collaborative data for infant formula powder soy-based

	Cis		*Trans*		Total vitamin A as RE (retinol equivalents)	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	14.05	14.19	48.05	47.17	62.10	61.36
Laboratory 2	14.28	14.16	50.80	49.35	65.08	63.51
Laboratory 3	13.08	13.32	47.81	49.78	60.89	63.10
Laboratory 4	15.58	14.63	53.36	41.23	68.94	55.87
Laboratory 5	14.91	14.80	52.28	52.76	67.19	67.57
Laboratory 6	12.36	11.77	47.09	47.36	59.45	59.13
Laboratory 7	15.21	14.34	53.56	52.78	68.77	67.12
Laboratory 8	13.47	15.64	48.68	42.63	62.15	58.27
Laboratory 9	9.56	13.40	33.79	48.50	43.35	61.90
Laboratory 10	17.19	16.56	54.44	57.11	71.64	73.67
Laboratory 11	14.35	14.95	51.41	51.58	65.76	66.53
Laboratory 12	12.80	13.15	49.71	47.96	62.51	61.11
Laboratory 13	11.26	10.66	44.58	44.74	55.84	55.40
Laboratory 14	14.55	14.00	52.77	52.72	67.31	66.72
Laboratory 15	11.64	12.11	45.00	45.77	56.64	57.88

Table 13. Collaborative data for adult nutritional powder milk protein-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	12.62	13.11	0.55	0.50	8.88	9.16
Laboratory 2	12.97	13.13	0.59	0.59	9.14	9.25
Laboratory 3	13.97	13.62	0.60	0.60	9.81	9.58
Laboratory 4	12.23	12.07	0.65	0.64	8.69	8.57
Laboratory 5	12.55	12.47	0.56	0.55	8.83	8.77
Laboratory 6	12.68	12.67	0.59	0.61	8.95	8.95
Laboratory 7	13.10	13.44	0.56	0.55	9.20	9.42
Laboratory 8	12.81	13.29	0.56	0.64	9.01	9.39
Laboratory 9	12.59	11.22	0.58	0.52	8.88	7.91
Laboratory 10	14.32	12.47	0.54	0.54	10.01	8.77
Laboratory 11	12.52	12.48	0.59	0.60	8.84	8.82
Laboratory 12	12.42	12.49	0.61	0.62	8.78	8.84
Laboratory 13	14.03	13.98	0.65	0.63	9.89	9.84
Laboratory 14	6.85[a]	6.84[a]	0.99[a]	1.01[a]	5.33[a]	5.33[a]
Laboratory 15	10.18	10.95	0.44	0.47	7.16	7.69

[a] Cochran and/or Grubbs outliers.

Table 14. Collaborative data for infant formula powder partially hydrolyzed soy-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.75	1.76	0.49[a]	0.40[a]	1.53	1.48
Laboratory 2	1.97	1.97	0.49	0.12	1.69	1.41
Laboratory 3	1.93	1.91	0.49[a]	0.07[a]	1.65	1.34
Laboratory 4	1.89	1.87	0.55	0.55	1.68	1.66
Laboratory 5	1.85	1.86	0.48	0.49	1.60	1.62
Laboratory 6	1.93	1.91	0.50	0.47	1.66	1.63
Laboratory 7	2.14	1.90	0.45	0.45	1.77	1.61
Laboratory 8	1.88	1.84	0.51	0.50	1.63	1.60
Laboratory 9	1.30	1.39	0.39	0.39	1.16	1.22
Laboratory 10	1.77	1.76	0.48	0.48	1.54	1.54
Laboratory 11	1.84	1.85	0.49	0.49	1.60	1.60
Laboratory 12	1.98	2.04	0.51	0.51	1.70	1.75
Laboratory 13	1.95	1.72	0.53	0.54	1.70	1.55
Laboratory 14	1.55	1.49	0.82[a]	0.79[a]	1.64	1.58
Laboratory 15	1.48	1.53	0.36	0.37	1.26	1.30

[a] Cochran and/or Grubbs outliers.

Table 15. Collaborative data for SRM 1849a

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	159.60	164.40	34.20	31.50	132.33	133.56
Laboratory 2	181.50	182.30	36.30	37.30	148.58	149.85
Laboratory 3	177.00	173.58	38.44	36.09	147.14	143.11
Laboratory 4	177.10	176.60	41.00	41.80	149.10	149.35
Laboratory 5	170.52	172.62	34.31	38.23	139.74	144.05
Laboratory 6	170.42	177.70	31.57	25.79	137.65	138.27
Laboratory 7	166.42	170.06	36.14	34.52	138.34	139.59
Laboratory 8	177.50	177.66	39.25	41.48	148.07	149.83
Laboratory 9	158.63[a]	136.08[a]	36.43	30.64	133.33[a]	113.93[a]
Laboratory 10	169.36	163.34	34.99	36.42	139.46	136.48
Laboratory 11	171.10	170.44	39.25	41.29	143.78	144.84
Laboratory 12	186.39	173.61	35.97	34.82	151.61	142.19
Laboratory 13	211.37	211.62	46.09	43.25	175.85[a]	173.92[a]
Laboratory 14	145.40	142.57	65.24[a]	58.61[a]	145.72	138.92
Laboratory 15	161.44	159.37	29.85	31.82	130.36[a]	130.42[a]

[a] Cochran and/or Grubbs outliers.

Table 16. Collaborative data for adult nutritional powder low fat

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	1.70	1.82	0.23[a]	0.13[a]	1.32	1.32
Laboratory 2	1.90	1.94	0.09	0.12	1.34	1.39
Laboratory 3	1.96	1.98	0.11	0.10	1.40	1.41
Laboratory 4	1.79	1.88	0.17	0.19	1.32	1.40
Laboratory 5	1.89	1.81	0.25	0.24	1.46	1.39
Laboratory 6	1.93	1.93	0.05	0.06	1.33	1.33
Laboratory 7	1.71	1.72	0.13	0.13	1.24	1.25
Laboratory 8	1.91	1.97	0.15	0.18	1.40	1.45
Laboratory 9	1.71[a]	0.95[a]	0.11	0.08	1.23[a]	0.70[a]
Laboratory 10	1.84	1.90	0.14	0.14	1.34	1.37
Laboratory 11	1.98	1.96	0.13	0.15	1.42	1.43
Laboratory 12	1.68	1.67	0.10	0.09	1.20	1.19
Laboratory 13	1.95	1.99	0.26	0.26	1.50	1.53
Laboratory 14	1.44	1.45	0.16	0.17	1.08	1.09
Laboratory 15	1.52[a]	1.31[a]	0.07	0.08	1.07	0.94

[a] Cochran and/or Grubbs outliers.

Table 17. Collaborative data for infant elemental powder

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	1.77	1.75	0.41	0.29	1.49	1.39
Laboratory 2	1.83	1.85	0.37	0.35	1.51	1.50
Laboratory 3	1.88	1.93	0.52	0.40	1.64	1.59
Laboratory 4	1.80	1.84	0.41	0.36	1.51	1.50
Laboratory 5	1.78	1.87	0.40	0.47	1.49	1.60
Laboratory 6	1.78	1.94	0.29	0.38	1.41	1.58
Laboratory 7	1.76	1.91	0.42	0.52	1.49	1.67
Laboratory 8	1.85	1.87	0.40	0.43	1.54	1.57
Laboratory 9	1.76	1.76	0.52	0.42	1.57	1.49
Laboratory 10	1.76	1.83	0.31	0.52	1.41	1.62
Laboratory 11	1.75	1.64	0.31	0.32	1.40	1.34
Laboratory 12	2.00	1.91	0.42	0.48	1.66	1.64
Laboratory 13	1.78	1.85	0.41	0.38	1.49	1.52
Laboratory 14	1.49	1.53	0.84[a]	0.58[a]	1.62	1.46
Laboratory 15	1.57	1.69	0.52	0.47	1.44	1.48

[a] Cochran and/or Grubbs outliers.

Table 18. Collaborative data for infant formula powder milk-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.36	1.36	0.47	0.46	1.26	1.25
Laboratory 2	1.50	1.52	0.47	0.47	1.35	1.36
Laboratory 3	1.51	1.48	0.49	0.49	1.38	1.35
Laboratory 4	1.45	1.45	0.53	0.52	1.36	1.36
Laboratory 5	1.47	1.48	0.52	0.51	1.37	1.37
Laboratory 6	1.43	1.43	0.43	0.45	1.28	1.29
Laboratory 7	1.45	1.42	0.50	0.48	1.34	1.31
Laboratory 8	1.45	1.44	0.50	0.49	1.34	1.33
Laboratory 9	1.07[a]	1.37[a]	0.39[a]	0.50[a]	1.00[a]	1.29[a]
Laboratory 10	1.39	1.37	0.49	0.50	1.29	1.29
Laboratory 11	1.45	1.49	0.49	0.51	1.33	1.38
Laboratory 12	1.55	1.57	0.51	0.51	1.41	1.43
Laboratory 13	1.41	1.48	0.52	0.54	1.33	1.39
Laboratory 14	1.17[a]	1.15[a]	0.82[a]	0.81[a]	1.39	1.36
Laboratory 15	1.30	1.33	0.45	0.45	1.21	1.22

[a] Cochran and/or Grubbs outliers.

Table 19. Collaborative data for infant formula RTF milk-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF	μg/100 g RTF
Laboratory 1	1.49	1.48	0.39	0.36	1.29	1.26
Laboratory 2	1.64	1.62	0.45	0.44	1.43	1.41
Laboratory 3	1.66	1.64	0.47	0.47	1.46	1.44
Laboratory 4	1.55	1.56	0.50	0.50	1.41	1.42
Laboratory 5	1.53	1.53	0.49	0.50	1.39	1.39
Laboratory 6	1.59	1.58	0.42	0.41	1.37	1.37
Laboratory 7	1.57	1.55	0.49	0.49	1.42	1.40
Laboratory 8	1.56	1.55	0.47	0.47	1.39	1.39
Laboratory 9	1.29[a]	1.47[a]	0.43	0.46	1.18[a]	1.32[a]
Laboratory 10	1.49	1.50	0.47	0.47	1.35	1.35
Laboratory 11	1.57	1.57	0.45	0.45	1.39	1.38
Laboratory 12	1.60	1.59	0.43	0.43	1.39	1.38
Laboratory 13	1.65	1.65	0.47	0.43	1.45	1.42
Laboratory 14	1.26[a]	1.28[a]	0.75[a]	0.76[a]	1.40	1.42
Laboratory 15	1.43	1.44	0.43	0.42	1.28	1.28

[a] Cochran and/or Grubbs outliers.

Table 20. Collaborative data for infant formula powder soy-based

	α-Tocopherol acetate		α-Tocopherol		Total vitamin E as TE	
Laboratory No.	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Laboratory 1	1.27	1.26	0.41	0.40	1.15	1.14
Laboratory 2	1.38	1.40	0.43	0.43	1.25	1.26
Laboratory 3	1.36	1.37	0.45	0.46	1.24	1.26
Laboratory 4	1.33	1.34	0.52	0.40	1.27	1.19
Laboratory 5	1.30	1.33	0.49	0.49	1.23	1.26
Laboratory 6	1.36	1.31	0.46	0.44	1.25	1.20
Laboratory 7	1.33	1.33	0.50	0.49	1.26	1.25
Laboratory 8	1.30	1.31	0.45	0.41	1.21	1.18
Laboratory 9	0.88[a]	1.27[a]	0.35	0.48	0.85[a]	1.21[a]
Laboratory 10	1.28	1.28	0.50	0.49	1.22	1.22
Laboratory 11	1.33	1.32	0.47	0.48	1.24	1.24
Laboratory 12	1.37	1.44	0.46	0.50	1.26	1.33
Laboratory 13	1.30	1.33	0.49	0.51	1.24	1.27
Laboratory 14	1.07	1.09	0.81[a]	0.80[a]	1.32	1.32
Laboratory 15	1.22	1.17	0.44	0.43	1.15	1.11

[a] Cochran and/or Grubbs outliers.

Recommendations

The completed AOAC INTERNATIONAL *Interlaboratory Study Workbook Revision 2.1* along with a statistical report (5) and a draft copy of the study report summarizing the outcomes of this collaborative study were submitted with the recommendation that AOAC First Action Method **2012.10** be accepted as a SPIFAN-endorsed AOAC Final Action Method. The ERP evaluated the collaborative study data in September 2014 and endorsed the recommendation, which was subsequently approved by the Official Methods Board in November 2014.

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Sabine Meng Jensen, Eurofins Steins Laboratorium A-S, Denmark (Vejen, Denmark)

Brendon Gill, Fonterra, New Zealand (Waikato, New Zealand)

Raquel De Guzman and Litz Rael, Mead Johnson Nutrition, Philippines (Manila, Philippines)

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