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Infant formula and adult nutritionals — Determination of vitamin E and vitamin A by normal phase high performance liquid chromatography

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National foreword

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**Infant formula and adult
nutritionals — Determination of
vitamin E and vitamin A by normal
phase high performance liquid
chromatography**

*Formules infantiles et produits nutritionnels pour adultes —
Détermination de la teneur en vitamine E et de la teneur en vitamine A
par chromatographie liquide à haute performance en phase normale*





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Foreword

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The committee responsible for this document is ISO/TC 34, *Food products* in collaboration with AOAC INTERNATIONAL. It is being published by ISO and separately by AOAC INTERNATIONAL. The method described in this International Standard is equivalent to the AOAC Official Method 2012-10: *Infant formula and adult nutritionals — Determination of vitamin E and vitamin A by normal phase high performance liquid chromatography*.

Infant formula and adult nutritionals — Determination of vitamin E and vitamin A by normal phase high performance liquid chromatography

WARNING — The use of this International Standard can involve hazardous materials, operations and equipment. This International Standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard specifies a method for the simultaneous quantitative determination of vitamin E (α -tocopherol and α -tocopheryl acetate) and vitamin A (13-*cis* and all-*trans* isomers of retinyl palmitate and retinyl acetate) present in all forms of infant and adult formulas (powders, ready-to-feed liquids and liquid concentrates).

Retinol is not used for fortification purposes and therefore is not addressed in this method. The innate amount in products is insignificant.

Stereoisomers of vitamin E, α -tocopherol and α -tocopheryl acetate, are not differentiated in this method.

2 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

2.1

adult nutritional

nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment, made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein

2.2

infant formula

breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding

[SOURCE: Codex Standard 72-1981]

3 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. The extract is analysed by normal phase HPLC using an analytical column with gradient elution. Quantification of α -tocopherol and α -tocopheryl acetate is done using fluorescence detection with excitation and emission wavelengths at 280 nm and 310 nm. Retinyl palmitate (*cis* and *trans*) and retinyl acetate (*cis* and *trans*) are quantified using UV detection at 325 nm.

4 Reagents and materials

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

4.1 Methyl-*t*-butyl ether, also known as *tert*-butylmethylether, HPLC grade.

4.2 *n*-Hexane, HPLC grade.

4.3 Ethanol, HPLC grade.

4.4 Methanol, HPLC grade.

4.5 Iso-octane (2,2,4-trimethylpentane), HPLC grade.

4.6 Papain (from *Carica papaya*), ≥ 3 U/mg, Sigma 76220¹⁾ or equivalent.

4.7 Hydroquinone, Sigma H9003¹⁾ or equivalent.

4.8 Glacial acetic acid, analytical reagent grade.

4.9 Anhydrous sodium acetate.

4.10 Dilute hydrochloric acid solution.

Dilute 100 ml of a hydrochloric acid solution with a mass fraction of 36 % to 200 ml with water.

4.11 Papain solution, mass concentration $\rho = 20$ g/l.

Dissolve 100 mg hydroquinone and 4 g anhydrous sodium acetate in approximately 80 ml of water in a 100 ml one-mark volumetric flask (5.11). Adjust the pH to 5,0 with dilute hydrochloric acid solution (4.10). Add 2 g of papain and make up to volume. Prepare fresh prior to use.

4.12 Acidified methanol solution.

Add 20 ml of glacial acetic acid to 1 l of methanol and mix. Prepare fresh on the day of use.

4.13 HPLC mobile phase A.

n-Hexane, filtered and degassed for 15 min in an ultrasonic bath.

4.14 HPLC mobile phase B.

Mix 750 ml of *n*-hexane with 250 ml of methyl-*t*-butyl ether. Add 3 ml of methanol, filter and degas for 15 min in an ultrasonic bath.

4.15 Standard substances

4.15.1 Retinyl palmitate reference standard, primary reference standard. The standard shall contain antioxidant. CAS 78-81-2.

1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.15.2 Retinyl acetate reference standard, primary reference standard. CAS 127-47-9.

4.15.3 α -tocopheryl acetate reference standard, primary reference standard. CAS 7695-91-2

4.15.4 α -tocopherol reference standard, primary reference standard. CAS 10191-41-0.

4.16 Standard solutions

4.16.1 Retinyl palmitate stock standard solution.

Weigh to the nearest 0,01 mg, approximately 70 mg of retinyl palmitate (4.15.1) into a 50 ml volumetric flask (5.11). Dissolve in and dilute to volume with iso-octane (4.5).

4.16.2 Retinyl acetate stock standard solution.

Weigh, to the nearest 0,01 mg, approximately 35 mg of retinyl acetate (4.15.2) into a 50 ml volumetric flask (5.11). Dissolve in and dilute to volume with ethanol (4.3).

4.16.3 α -tocopheryl acetate stock standard solution.

Weigh, to the nearest 0,01 mg, approximately 180 mg of α -tocopheryl acetate (4.15.3) into a 50 ml volumetric flask (5.11). Dissolve in and dilute to volume with iso-octane.

4.16.4 α -tocopherol stock standard solution.

Weigh, to the nearest 0,01 mg, approximately 100 mg of α -tocopherol (4.15.4) into a 50 ml volumetric flask (5.11). Dissolve in and dilute to volume with iso-octane.

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

4.16.5 Combined working standard solution 1.

Transfer by pipette 4 ml of retinyl palmitate stock standard solution (4.16.1), 4 ml of retinyl acetate stock standard solution (4.16.2), 7 ml of α -tocopheryl acetate stock standard solution (4.16.3) and 20 ml of α -tocopherol stock standard solution (4.16.4), into a 50 ml volumetric flask (5.11) and dilute to volume with iso-octane. Prepare this solution freshly prior to use.

4.16.6 Combined working standard solution 2.

Transfer by pipette 8 ml of combined working standard solution 1 (4.16.5) into a 100 ml volumetric flask (5.11) and dilute to volume with iso-octane. Prepare this solution freshly prior to use.

4.16.7 Calibration standard solutions

Into separate 50 ml volumetric flasks (5.11), transfer by pipette 0,5 ml, 2 ml, 4 ml, 8 ml, 16 ml and 32 ml of combined working standard solution 2 (4.16.6), 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.

4.17 Stock standard purity determinations

4.17.1 Spectrometric purity of retinyl palmitate stock solution

Pipet 1 ml of retinyl palmitate stock standard solution (4.16.1) into a 100 ml volumetric flask (5.11) and make up to volume with ethanol. Determine the absorption at 325 nm, zeroed against ethanol in a 1 cm quartz cell. Repeat the reading a further two times, rinsing the sample cuvette with the solution before each reading. Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_{RP} , of retinyl palmitate using Formula (1):

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

where

- A is the average absorbance reading;
- 975 is the extinction coefficient of retinyl palmitate at 325 nm (see Reference [1]);
- m_{st} is the mass of the reference standard in mg.

4.17.2 Spectrometric purity of retinyl acetate stock solution

Pipet 1 ml of retinyl acetate stock standard solution (4.16.2), into a 100 ml volumetric flask (5.11) and make up to volume with ethanol. Determine the absorption at 325 nm, zeroed against ethanol in a 1 cm quartz cell. Repeat the reading a further two times, rinsing the sample cuvette with the solution before each reading. Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_{RA} , of retinyl acetate using Formula (2):

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

where

- A is the average absorbance reading;
- 1 560 is the extinction coefficient of retinyl acetate at 325 nm (see Reference [1]);
- m_{st} is the mass of the reference standard in mg.

4.17.3 Spectrometric purity of α -tocopheryl acetate stock solution

Pipet 3 ml of α -tocopheryl acetate stock standard solution (4.16.3), into a 100 ml volumetric flask (5.11) and make up to volume with ethanol. Determine the absorption at 284 nm, zeroed against ethanol in a 1 cm quartz cell. Repeat the reading a further two times, rinsing the sample cuvette with the solution before each reading. Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_{TA} , of α -tocopheryl acetate using Formula (3):

$$SP_{TA} = \frac{A}{43,6} \times \frac{50}{m_{st}} \times \frac{100}{3} \times 10 \quad (3)$$

where

- A is the average absorbance reading;
- 43,6 is the extinction coefficient of α -tocopheryl acetate at 284 nm (see Reference [1]);
- m_{st} is the mass of the reference standard in mg.

4.17.4 Spectrometric purity of α -tocopherol stock solution

Pipet 3 ml of α -tocopherol stock standard solution (4.16.4), into a 100 ml volumetric flask (5.11) and make up to volume with ethanol. Determine the absorption at 292 nm, zeroed against ethanol in a 1 cm quartz cell. Repeat the reading a further two times, rinsing the sample cuvette with the solution before each reading. Calculate the average absorbance reading. Calculate the spectrometric purity as a decimal, SP_T , of α -tocopherol using Formula (4):

$$SP_T = \frac{A}{75,8} \times \frac{50}{m_{st}} \times \frac{100}{3} \times 10 \quad (4)$$

where

- A is the average absorbance reading;
- 75,8 is the extinction coefficient of α -tocopherol at 292 nm (see Reference [1]);
- m_{st} is the mass of the reference standard in mg.

4.17.5 Chromatographic purity of stock standard solutions

Prepare each stock standard solution separately as follows.

Into four separate 100 ml volumetric flasks (5.11) transfer by pipette 1 ml of each of the stock standard solutions, retinyl palmitate (4.16.1), retinyl acetate (4.16.2), α -tocopheryl acetate (4.16.3) and α -tocopherol (4.16.4). Label each flask with the individual analyte names. Mix and dilute each to volume with iso-octane.

Into four separate labelled 2 ml auto sampler vials transfer by auto pipettor, 60 μ l of retinyl palmitate solution 30 μ l of retinyl acetate solution, 100 μ l of α -tocopheryl acetate solution and 400 μ l of α -tocopherol. Fill vial with iso-octane to approximately 2 ml.

Vortex briefly and inject into the liquid chromatography system according to the method parameters described in 6.2. Analyse retinyl palmitate and retinyl acetate by UV at 325 nm. For α -tocopheryl acetate, analyse by UV at 284 nm and for α -tocopherol, analyse at 292 nm.

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

$$CP = \frac{\text{Area of peak of interest}}{\text{Total peak area excluding solvent}} \quad (5)$$

Quantify the retinyl palmitate/acetate reference standard against the *trans* peak in the chromatogram only.

4.17.6 Calculation of the concentrations of the calibration 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 Formula (6) to Formula (9) in μ g/ml for retinyl palmitate (RP) and retinyl acetate (RA) and mg/ml for α -tocopherol (T) and α -tocopheryl acetate (TA).

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

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

$$\rho_{\text{WTA}} = SP_{\text{TA}} \times CP_{\text{TA}} \times \frac{m_{\text{st}}}{50} \times \frac{7}{50} \times \frac{8}{100} \times \frac{V_{\text{a}}}{50} \quad (8)$$

$$\rho_{\text{WT}} = SP_{\text{T}} \times CP_{\text{T}} \times \frac{m_{\text{st}}}{50} \times \frac{20}{50} \times \frac{8}{100} \times \frac{V_{\text{a}}}{50} \quad (9)$$

where

V_{a} is 0,5 ml, 2 ml, 4 ml, 8 ml, 16 ml and 32 ml respectively for the calibration levels;

m_{st} is the mass of the reference standard in mg;

SP is the UV spectrometric purity;

CP is the chromatographic purity as a decimal;

1 000 is the conversion factor from mg/ml to $\mu\text{g/ml}$.

5 Apparatus

Usual laboratory glassware and equipment and, in particular, the following.

5.1 HPLC system, consisting of a pump, autosampler, programmable UV detector operating at 325 nm for vitamin A and a fluorescence detector at an excitation wavelength of 280 nm and an emission wavelength for 310 nm for vitamin E.

5.2 HPLC column, analytical normal phase column, e.g. Agilent Zorbax® NH₂²⁾ (5 μm , 150 mm x 4,6 mm) or equivalent.

5.3 Water bath, set at 37 °C \pm 2 °C.

5.4 Centrifuge, with adaptors for 50 ml centrifuge tubes, capable of 4 000 rpm.

5.5 UV-VIS spectrometer, with 1 cm quartz cells.

5.6 Analytical balance, weighing to 4 decimal places.

5.7 Amber HPLC vials, 2 ml capacity, with plastic caps and polytetrafluoroethylene (PTFE) seals.

5.8 Disposable centrifuge tubes, 50 ml capacity.

5.9 Laboratory mechanical test tube shaker.

5.10 Sonicator bath.

5.11 One-mark volumetric flasks, capacity 50 ml and 100 ml.

5.12 Vacuum filtration apparatus, with 0,45 μm nylon membrane.

5.13 Laboratory glass bottles, capacity, 250 ml, 1 l and 2 l.

2) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.14 Pipettors and tips.

6 Procedure

6.1 Sample preparation

6.1.1 General

If the powder sample matrix is unknown, assume it is non-homogeneous and proceed with [6.1.2](#).

6.1.2 Dry blended powder samples

For dry blended/non-homogenous powder samples, accurately weigh approximately 25,0 g (m_1), into a 250 ml bottle ([5.13](#)). Dissolve using warm water (about 40 °C to 45 °C), cool and add 200 g of water. Note the final mass (m_2). Accurately weigh approximately 5,0 g (m_3) of reconstituted sample into a screw-top centrifuge tube. Calculate the sample mass (powder equivalent), m_s , using Formula (10):

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

6.1.3 Wet blended powder samples

For wet blended homogenous powder samples, accurately weigh approximately 0,525 g into a screw-top 50 ml centrifuge tube. Add 5 ml warm water of approximately 40 °C and shake to dissolve.

6.1.4 Liquid samples

For ready-to-feed samples or concentrated liquid products, accurately weigh approximately 5,0 g (m_3) of thoroughly homogenized sample into a screw-top 50 ml centrifuge tube.

6.1.5 Sample extraction

To the above weighed solutions, add 5 ml of papain solution ([4.11](#)). Mix to disperse each sample, cap, and place the tubes in a 37 °C ± 2 °C water bath for 20 min to 25 min. Remove the samples from the water bath and cool. Place in a freezer for about 5 min or refrigerate for about 20 min. Add 20 ml acidified methanol ([4.12](#)) to each sample tube and shake the tubes for 10 min preferably with a mechanical shaker.

Accurately pipet 10 ml of iso-octane to each sample tube. Close tightly to avoid leakage and shake the tubes for 10 min preferably with a mechanical shaker. Centrifuge for 10 min at 4 000 rpm to obtain a clear iso-octane layer. Transfer an aliquot of the clear iso-octane layer into amber vials for HPLC analysis.

6.2 HPLC analysis

6.2.1 General

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

Column: Agilent Zorbax® NH₂³) (5 µm, 150 mm × 4,6 mm);

Mobile phase A: *n*-hexane;

Mobile phase B: mixture of 750 ml of *n*-hexane, 250 ml of methyl-*t*-butyl ether and 3 ml of methanol;

3) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

Flow rate: 1,5 ml/min;

Injection volume: 50 µl;

Column oven: 40 °C ± 2 °C;

Run time: 20 min.

Examples of typical chromatograms are given in [Annex A](#).

6.2.2 Detector settings

Set the photo diode array (PDA)/UV detector at 325 nm for retinyl palmitate and retinyl acetate. Set the fluorescence detector at excitation wavelength of 280 nm and emission wavelengths of 310 nm for α-tocopheryl acetate and α-tocopherol.

6.2.3 Pump gradient elution cycle

The pump gradient elution cycle is given in [Table 1](#).

Table 1 — Pump gradient elution cycle

Time min	Flow rate ml/min	% mobile phase A	% mobile phase B
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

7 System suitability

The resolution between *cis* and *trans* retinyl palmitate and between *cis* and *trans* retinyl acetate should be checked when running this method; in the reference standard it should be > 1,5.

8 Calculations

Identify the all-*trans* and *cis* retinyl palmitate and acetate isomers, the α-tocopherol and the α-tocopheryl acetate peaks on the sample chromatogram by comparison with the retention time of the corresponding peaks in the standard solution.

Calculate the concentration, *w*, of the sample in µg/100 g for retinyl palmitate or retinyl acetate isomers and mg/100 g for α-tocopherol or α-tocopheryl acetate (powder or liquid) in accordance with Formula (11):

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

where

- A* is the peak area or height of retinyl palmitate/acetate (13-*cis* plus all-*trans*) or α-tocopherol or α-tocopheryl acetate in the test sample solution;
- I* is the intercept of the calibration curve for each analyte;

- S is the slope of the calibration curve for each analyte;
- V_{iso} is the volume of iso-octane used (here: $V_{\text{iso}} = 10$ ml);
- 100 is the factor to convert in 100 g basis;
- m_s is the 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 *trans*-isomers to the total retinyl palmitate/acetate activity.

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

Convert vitamin A results to retinol using stoichiometric calculations in accordance with Formula (12):

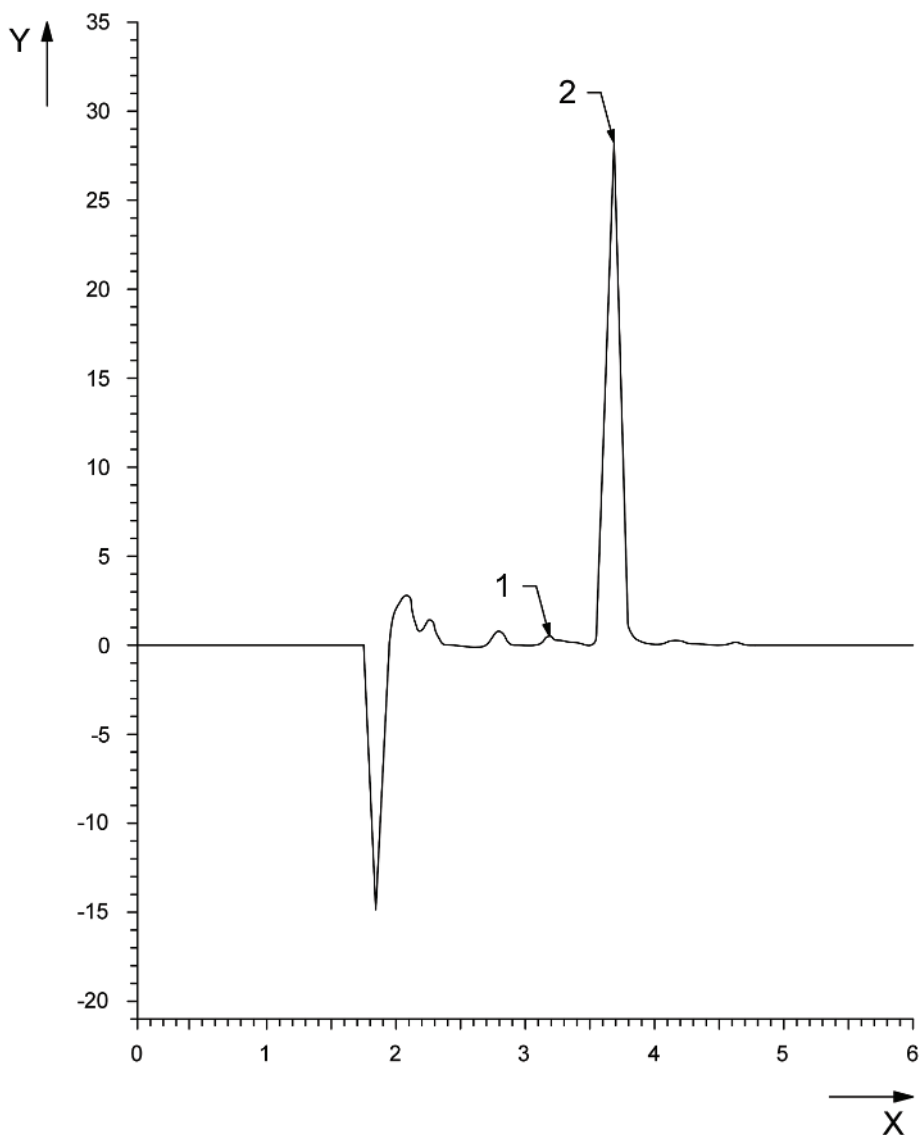
$$\text{Vitamin A as retinol } (\mu\text{g}/100 \text{ g}) = (\text{retinyl palmitate in } \mu\text{g}/100 \text{ g} \times 0,55) + (\text{retinyl acetate } \mu\text{g}/100 \text{ g} \times 0,87) \quad (12)$$

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).

Annex A (informative)

Examples of chromatograms



Key

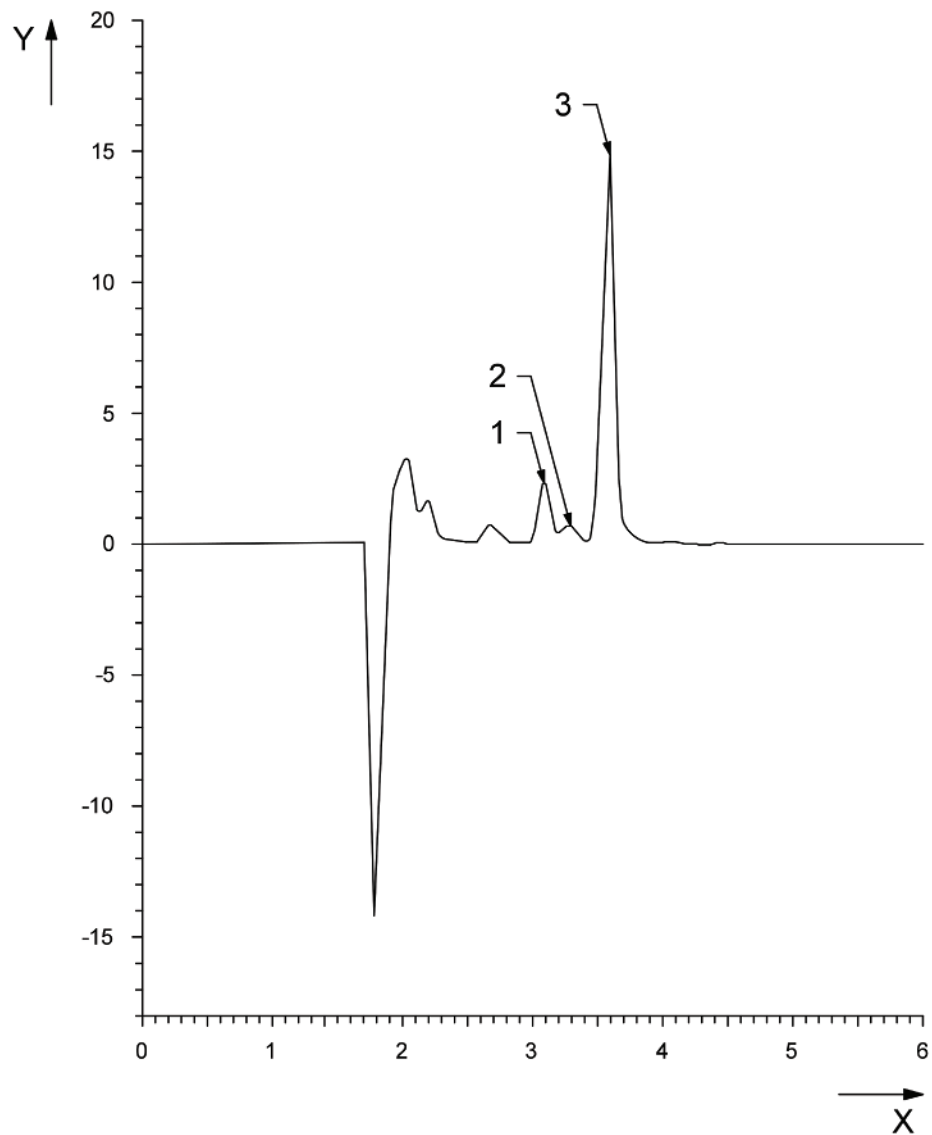
Peak 1 13-*Cis* isomer

Peak 2 *Trans* isomer

X time in min

Y milli Absorbance Units (mAU)

Figure A.1 — HPLC chromatogram of retinyl palmitate calibration standard

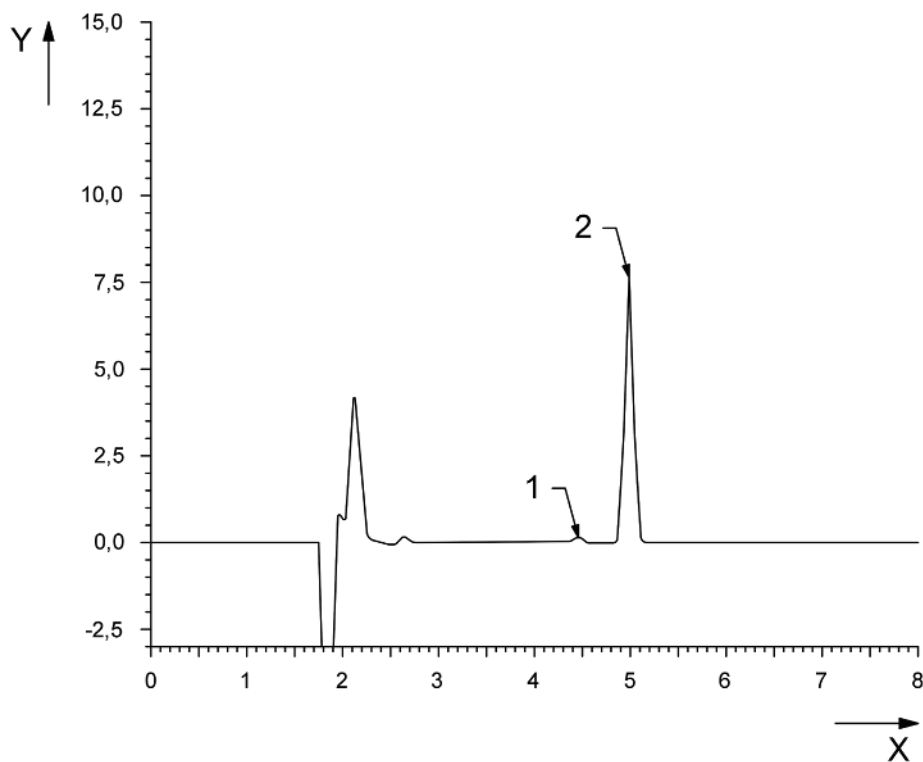


Key

Peak 1 13-*Cis* isomer
Peak 2 *Cis* isomer
Peak 3 *Trans* isomer

X time in min
Y milli Absorbance Units (mAU)

Figure A.2 — HLPC chromatogram of retinyl palmitate test sample

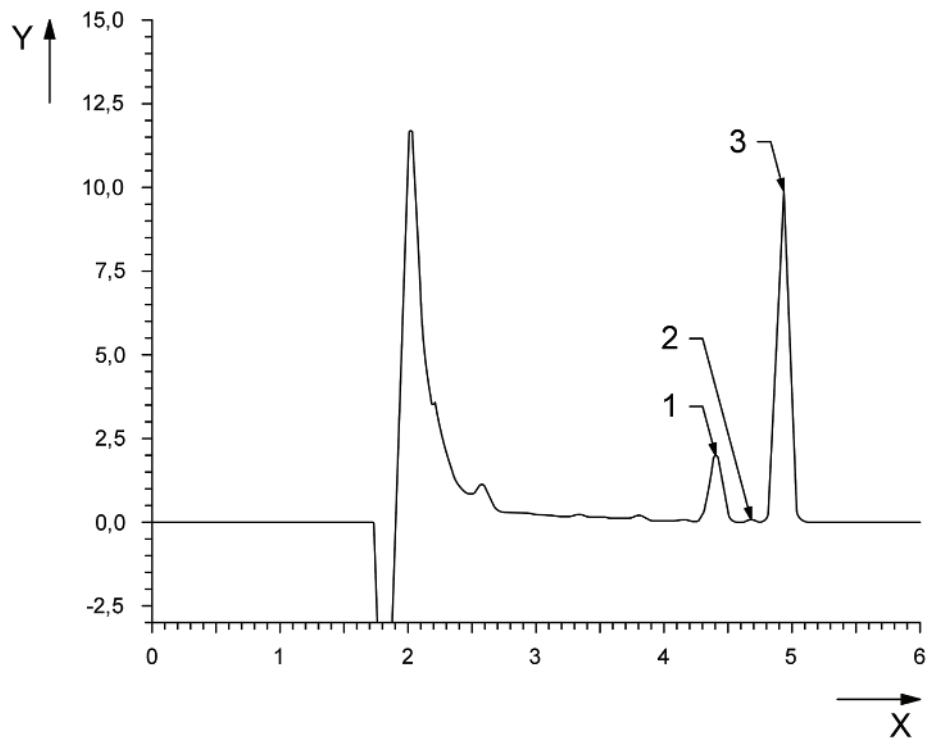


Key

Peak 1 13-*Cis* isomer
Peak 2 *Trans* isomer

X time in min
Y milli Absorbance Units (mAU)

Figure A.3 — HLPC chromatogram of retinyl acetate calibration standard



Key

Peak 1 13-*Cis* isomer

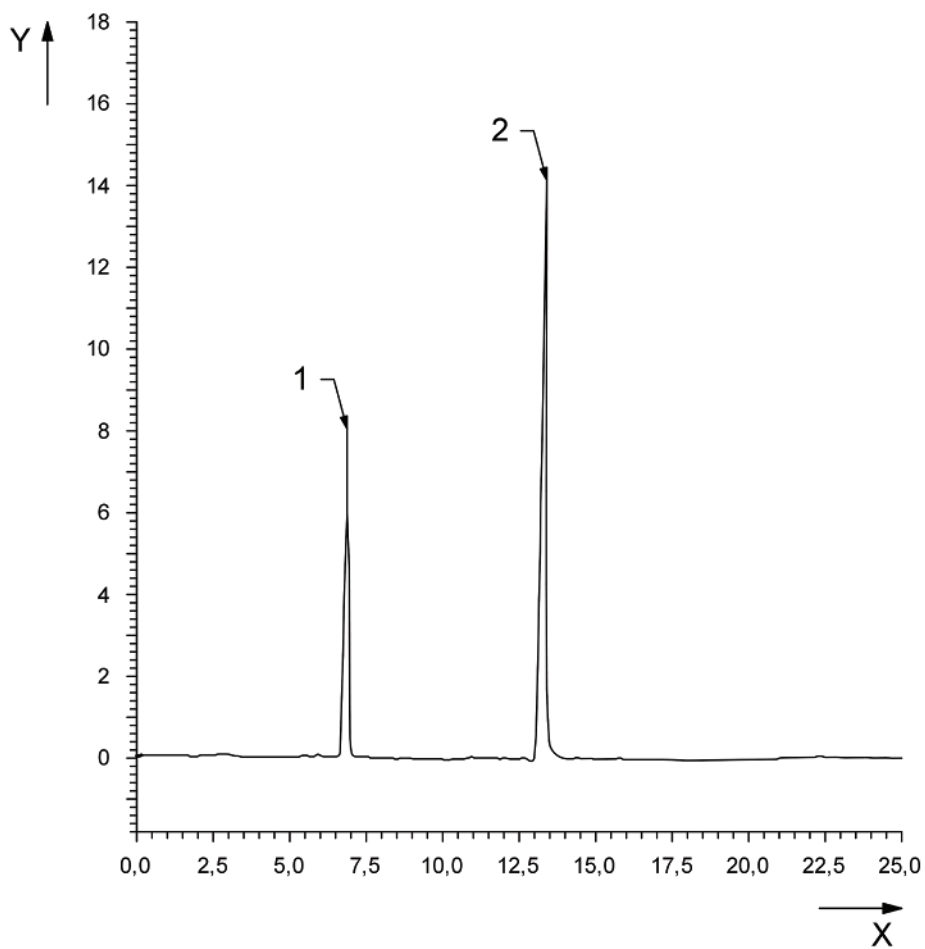
Peak 2 *Cis* isomer

Peak 3 *Trans* isomer

X time in min

Y milli Absorbance Units (mAU)

Figure A.4 — HPLC chromatogram of retinyl acetate test sample



Key

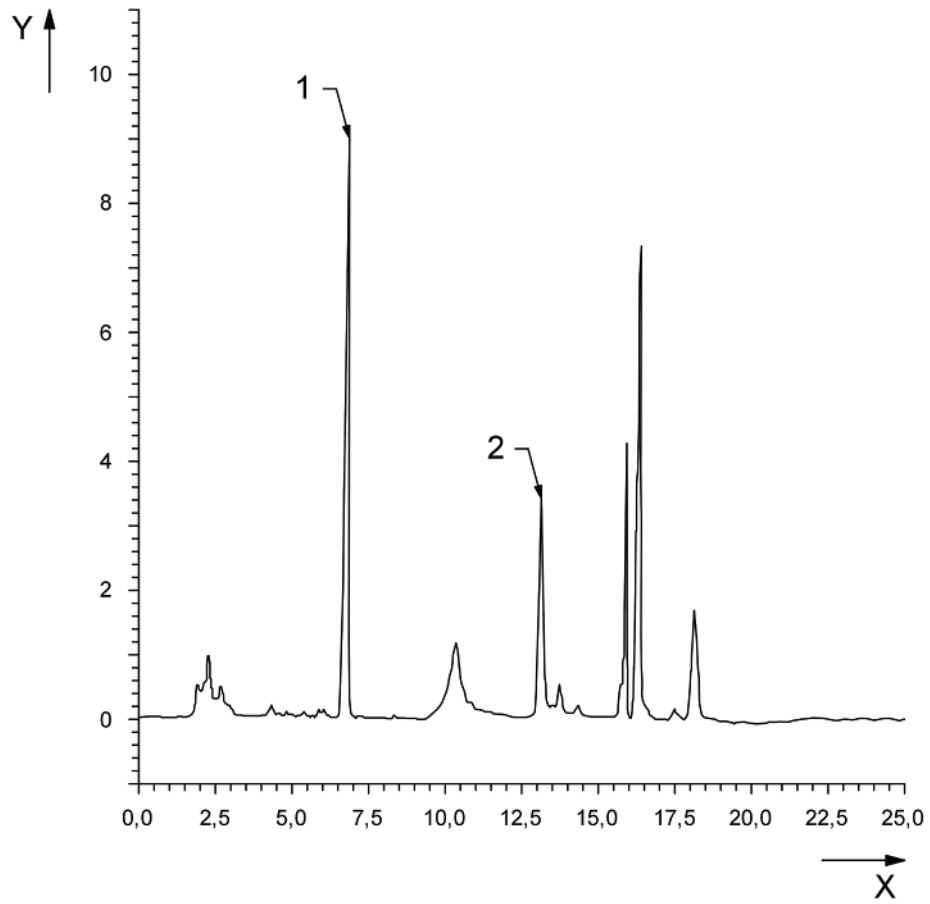
Peak 1 α -tocopheryl acetate

Peak 2 α -tocopherol

X time in min

Y milli Absorbance Units (mAU)

Figure A.5 — HPLC chromatogram of α -tocopheryl acetate and α -tocopherol calibration standard



Key

Peak 1 α -tocopheryl acetate

X time in min

Peak 2 α -tocopherol

Y milli Absorbance Units (mAU)

Figure A.6 — HPLC chromatogram of α -tocopheryl acetate and α -tocopherol sample chromatogram

Annex B (informative)

Precision data

The data given in [Tables B.1, B.2](#) and [B.3](#) were obtained in an interlaboratory study and published in 2015[2], in accordance with ISO 5725-2[3] and AOAC-IUPAC Harmonized Protocol for collaborative study procedures, to assess precision characteristics of a method of analysis.[4] The study was performed based on requirements given in References [5] and [6].

More information on the validation of the method can be found at <http://standards.iso.org/iso/20633>

Table B.1 — Precision data for retinyl acetate, (1^a and 2^b), and retinyl palmitate, (3^c to 8^h) expressed as retinol

Sample	1 ^a	2 ^b	3 ^{c, j}	4 ^d	5 ^e	6 ^f	7 ^g	8 ^h
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
Number of laboratories	15	15	15	15	15	15	15	15
Number of non-compliant laboratories	0	0	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	15	12	15	15	15	14	14	15
Number of outliers (laboratories)	0	3	0	0	0	1	1	0
Number of accepted results	30	24	30	30	30	28	28	30
Mean value, \bar{x} , $\mu\text{g}/100\text{ g RTF}^i$	46,34	67,39	6,49	47,55	62,56	66,58	57,34	48,35
Repeatability standard deviation s_r , $\mu\text{g}/100\text{ g RTF}$	7,69	1,55	0,21	7,50	4,28	0,75	0,61	7,31
Reproducibility standard deviation s_R , $\mu\text{g}/100\text{ g RTF}$	10,48	8,04	0,52	10,33	6,04	4,33	4,13	7,86
Coefficient of variation of repeatability, $C_{V,r}$, %	16,60	2,30	3,26	15,78	6,84	1,13	1,06	15,13
Coefficient of variation of reproducibility, $C_{V,R}$, %	22,61	11,93	8,02	21,73	9,66	6,51	7,20	16,25
Repeatability limit r [$r = 2,8 \times s_r$], $\mu\text{g}/100\text{ g RTF}$	21,54	4,34	0,59	21,01	11,98	2,10	1,70	20,48
Reproducibility limit R [$R = 2,8 \times s_R$], $\mu\text{g}/100\text{ g RTF}$	29,34	22,52	1,46	28,92	16,91	12,13	11,56	21,99
HorRat value, according to Reference [7]	1,25	0,69	0,33	1,20	0,56	0,38	0,41	0,91

^a Adult nutritional powder milk protein based, ^b Infant formula powder partially hydrolysed soy based, ^c SRM 1849a, ^d Adult nutritional powder low fat, ^e Infant formula powder soy based, ^f Infant formula powder milk based, ^g Infant formula RTF milk based, ^h Infant elemental powder, ⁱ RTF is ready-to-feed, ^j mg/kg powder

NOTE 1 The results are expressed as μg retinol per 100 g RTF.

NOTE 2 SRM 1849a values determined are lower than the certified values, possibly due to inadequate storage conditions during the collaborative studies. The certified value ($7,68\text{ mg/kg} \pm 0,23\text{ mg/kg}$) was obtained during a single lab validation study for this material, see Reference [10].

Table B.2 — Precision data for vitamin E (α -tocopherol)

Sample	1 ^a	2 ^b	3 ^{c,j}	4 ^d	5 ^e	6 ^f	7 ^g	8 ^h
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
Number of laboratories	15	15	15	15	15	15	15	15
Number of non-compliant laboratories	0	0	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	14	11	14	14	14	13	14	14
Number of outliers (laboratories)	1	4	1	1	1	0	1	1
Number of accepted results	28	22	28	28	28	26	28	28
Mean value, \bar{x} , mg/100 g RTF ⁱ	0,58	0,48	36,38	0,14	0,46	0,49	0,45	0,41
Repeatability standard deviation s_r , mg/100 g RTF	0,023	0,008	2,103	0,012	0,036	0,008	0,012	0,064
Reproducibility standard deviation s_R , mg/100 g RTF	0,052	0,057	4,539	0,061	0,040	0,279	0,035	0,072
Coefficient of variation of repeatability, $C_{V,r}$, %	3,99	1,67	5,78	8,90	7,89	1,57	2,59	15,48
Coefficient of variation of reproducibility, $C_{V,R}$, %	9,10	11,94	12,47	43,56	8,74	5,68	7,73	17,44
Repeatability limit r [$r = 2,8 \times s_r$], $\mu\text{g}/100 \text{ g RTF}$	0,064	0,022	5,889	0,035	0,101	0,022	0,033	0,178
Reproducibility limit R [$R = 2,8 \times s_R$], $\mu\text{g}/100 \text{ g RTF}$	0,147	0,159	12,71	0,170	0,112	0,078	0,098	0,201
HorRat value, according to Reference [Z]	0,930	0,920	1,870	2,180	0,680	0,440	0,600	1,340

^a Adult nutritional powder milk protein based, ^b Infant formula powder partially hydrolysed soy based, ^c SRM 1849a, ^d Adult nutritional powder low fat, ^e Infant formula powder soy based, ^f Infant formula powder milk based, ^g Infant formula RTF milk based, ^h Infant elemental powder, ⁱ RTF is ready-to-feed, ^j mg/kg powder

Table B.3 — Precision data for vitamin E (α -tocopheryl acetate)

Sample	1 ^a	2 ^b	3 ^{c, j}	4 ^d	5 ^e	6 ^f	7 ^g	8 ^h
Year of interlaboratory test	2014	2014	2014	2014	2014	2014	2014	2014
Number of laboratories	15	15	15	15	15	15	15	15
Number of non-compliant laboratories	0	0	0	0	0	0	0	0
Number of laboratories retained after eliminating outliers	14	15	14	13	14	13	13	15
Number of outliers (laboratories)	1	0	0	0	0	2	2	0
Number of accepted results	28	30	28	26	28	26	26	30
Mean value, \bar{x} , mg/100 g RTF ⁱ	12,73	1,80	172,89	1,84	1,30	1,44	1,56	1,79
Repeatability standard deviation s_r , mg/100 g RTF	0,489	0,066	3,374	0,388	0,022	0,021	0,009	0,061
Reproducibility standard deviation s_R , mg/100 g RTF	0,926	0,203	14,991	0,156	0,084	0,067	0,065	0,119
Coefficient of variation of repeatability, $C_{V,r}$, %	3,84	3,65	1,95	2,11	1,67	1,43	0,60	3,38
Coefficient of variation of reproducibility, $C_{V,R}$, %	7,28	11,25	8,67	8,50	6,47	4,62	4,15	6,66
Repeatability limit r [$r = 2,8 \times s_r$], $\mu\text{g}/100 \text{ g RTF}$	1,369	0,184	9,447	0,109	0,061	0,058	0,026	0,170
Reproducibility limit R [$R = 2,8 \times s_R$], $\mu\text{g}/100 \text{ g RTF}$	2,594	0,567	41,976	0,437	0,236	0,187	0,181	0,334
HorRat value, according to Reference [Z]	0,330	1,070	0,580	0,810	0,580	0,420	0,380	0,630

^a Adult nutritional powder milk protein based, ^b Infant formula powder partially hydrolysed soy based, ^c SRM 1849a, ^d Adult nutritional powder low fat, ^e Infant formula powder soy based, ^f Infant formula powder milk based, ^g Infant formula RTF milk based, ^h Infant elemental powder, ⁱ RTF is ready-to-feed, ^j mg/kg powder

Annex C (informative)

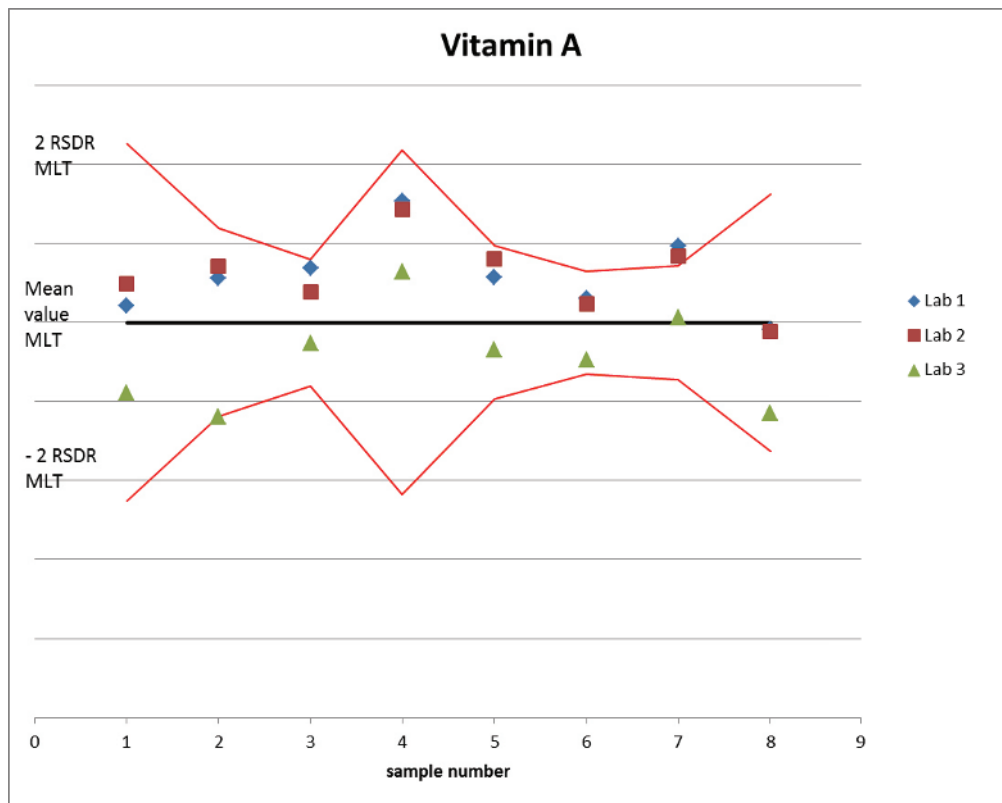
Comparison between AOAC 2012.10, EN 12822 and EN 12823-1

In order to ensure comparability of the data generated by EN 12822[8], EN 12823-1[9] and AOAC 2012.10[10], data were collected by analysing the same samples with all methods. The results of this comparison are given in [Table C.1](#) and [Table C.2](#) and illustrated in [Figure C.1](#) and [Figure C.2](#).

Table C.1 — Comparison of results for vitamin A

Sample	AOAC 2012.10	AOAC 2012.10	EN 12823-1	EN 12823-1	EN 12823-1
	<i>n</i> = 11 to 15 laboratories	<i>n</i> = 11 to 15 laboratories	Lab 1	Lab 2	Lab 3
	µg/100 g RTF ^a	<i>C_{V,R}</i> , %	µg/100 g RTF	µg/100 g RTF	µg/100 g RTF
Adult nutritional powder, milk protein based	46,34	22,61	48,29	50,84	38,07
Infant formula powder, partially hydrolysed, soy based	67,39	11,93	74,93	76,92	51,28
SRM 1849a	6,49 ^b	8,02	7,38	6,99	6,14
Adult nutritional powder, low fat	47,55	21,73	62,16	61,16	53,61
Infant formula powder, soy based	62,56	9,66	69,71	72,59	58,16
Infant formula powder, milk based	66,58	6,51	70,60	69,71	60,27
Infant formula powder RTF, milk based	57,34	7,20	68,35	67,00	58,00
Infant elemental powder	48,35	16,25	47,51	47,29	37,30
a RTF = ready-to-feed					
b mg/kg powder					

In [Figure C.1](#), the values determined with EN 12823-1 by the participating three laboratories are plotted against $\pm 2 C_{V,R}$ as determined by the multi laboratory trial (MLT) for AOAC 2012.10 with 11 to 15 participating laboratories. The $2 C_{V,R}$ boundaries should include 95 % of the data based on AOAC 2012.10.



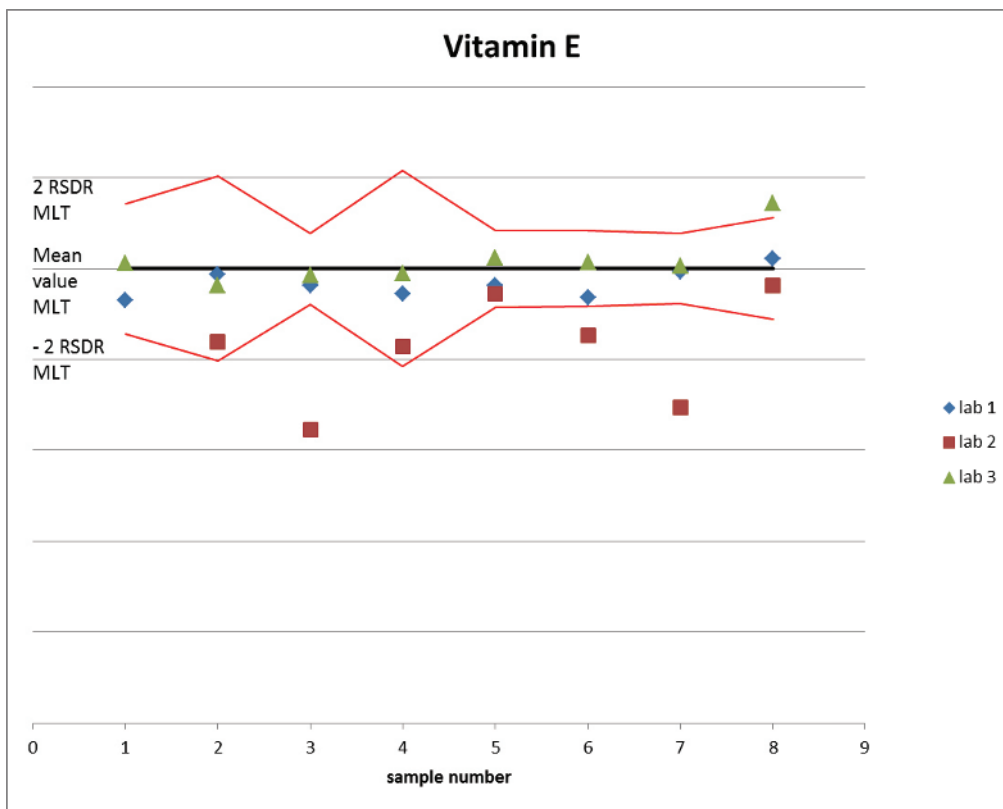
NOTE The figure illustrates the determination of vitamin A by EN 12823-1 with 3 participating laboratories plotted against 2 $C_{V,R}$ values as determined by AOAC 2012.10 with 11 to 15 laboratories. The values of the 3 participating laboratories are standardized against the mean value of the multi laboratory test for AOAC 2012.10.

Figure C.1 — Comparison of vitamin A determination

Table C.2 — Comparison of results for vitamin E (α -tocopheryl acetate plus α -tocopherol expressed as α -tocopherol)

Sample	AOAC 2012.10	AOAC 2012.10	EN 12822	EN 12822	EN 12822
	$n = 11$ to 15 laboratories	$n = 11$ to 15 laboratories	Lab 1	Lab 2	Lab 3
	mg/100 g RTF ^a	$C_{V,R}$, %	mg/100 g RTF	mg/100 g RTF	mg/100 g RTF
Adult nutritional powder, milk protein based	12,18	7,17	11,32	-	12,32
Infant formula powder, partially hydrolyzed, soy based	2,12	10,15	2,09	1,78	2,04
SRM 1849a	193,90 ^b	3,91	186,50	125,00	191,00
Adult nutritional powder, low fat	1,82	10,78	1,71	1,50	1,80
Infant formula powder, soy based	1,64	4,22	1,58	1,55	1,68
Infant formula powder, milk based	1,80	4,13	1,69	1,54	1,83
Infant formula powder RTF, milk based	1,87	3,84	1,86	1,30	1,88
Infant elemental powder	1,77	5,55	1,81	1,70	2,03
a RTF = ready-to-feed					
b mg/kg powder					

In [Figure C.2](#), the values determined with EN 12822 by the participating three laboratories are plotted against $\pm 2 C_{V,R}$ as determined by the multi laboratory (MLT) for AOAC 2012.10 with 11 to 15 participating laboratories. The $2 C_{V,R}$ boundaries should include 95 % of the data based on AOAC 2012.10 validation.



NOTE The figure illustrates the determination of vitamin E (α -tocopheryl acetate plus α -tocopheryl expressed as α -tocopheryl) by EN 12822 with 3 participating laboratories plotted against 2 $C_{V,R}$ values as determined by AOAC 2012.10 with 11 to 15 laboratories. The values of the 3 participating laboratories are standardized against the mean value of the multi laboratory test for AOAC 2012.10.

Figure C.2 — Comparison of vitamin E determination

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