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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 procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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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.*

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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.

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2 Terms and definitions

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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.

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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 76220[1)] or equivalent.

4.7 Hydroquinone, Sigma H9003[1)] or equivalent.

4.8 Glacial acetic acid, analytical reagent grade.

4.9 Anhydrous sodium acetate.

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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.

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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.

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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.

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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.

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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.

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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):

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$$SP_{RA} = \frac{A}{1\,560} \times \frac{50}{m_{st}} \times \frac{100}{1} \times 10 \quad (2)$$

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.

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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):

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

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.

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

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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):

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

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 µg/ml for retinyl palmitate (RP) and retinyl acetate (RA) and mg/ml for α-tocopherol (T) and α-tocopheryl acetate (TA).

$$\rho_{WRP} = SP_{RP} \times CP_{RP} \times \frac{m_{st}}{50} \times \frac{4}{50} \times \frac{8}{100} \times \frac{V_a}{50} \times 1\,000 \tag{6}$$

$$\rho_{WRA} = SP_{RA} \times CP_{RA} \times \frac{m_{st}}{50} \times \frac{4}{50} \times \frac{8}{100} \times \frac{V_a}{50} \times 1\,000 \tag{7}$$

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

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

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 µg/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.

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5.2 HPLC column, analytical normal phase column, e.g. Agilent Zorbax® NH_2[2)] (5 µm, 150 mm x 4,6 mm) or equivalent.

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5.3 Water bath, set at 37 °C ± 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.

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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):

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

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.

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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.

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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® NH_2[3)] (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;

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