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Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 1: Measurements of all-trans-retinol and 13-cis-retinol

Lebensmittel - Bestimmung von Vitamin A mit Hochleistungs-Flüssigchromatographie - Teil 1: Bestimmung von all-trans-Retinol und 13-cis-Retinol

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Produits alimentaires - Dosage de la vitamine A par chromatographie liquide haute performance - Partie 1: Dosage du tout-trans-rétinol et du 13-cis-rétinol

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SIST EN 12823-1:2000 **en**

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Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 1: Measurement of all-trans-retinol and 13-cis-retinol

Produits alimentaires - Dosage de la vitamine A par chromatographie liquide haute performance - Partie 1: Dosage du tout-trans-rétinol et du 13-cis-rétinol

Lebensmittel - Bestimmung von Vitamin A mit Hochleistungs-Flüssigchromatographie - Teil 1: Bestimmung von all-trans-Retinol und 13-cis-Retinol

This European Standard was approved by CEN on 2 January 2000.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

Central Secretariat: rue de Stassart, 36 B-1050 Brussels

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Foreword

This European Standard has been prepared by Technical Committee CEN/TC 275, Food analysis - Horizontal methods, the Secretariat of which is held by DIN.

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This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by August 2000, and conflicting national standards shall be withdrawn at the latest by August 2000.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

This European Standard, Foodstuffs - Determination of vitamin A by high performance liquid chromatography, consists of two parts:

Part 1: Measurement of all-trans-retinol and 13-cis-retinol;

Part 2: Measurement of β-carotene.

This European Standard provides the base for the analytical methods. It is intended to serve as a frame in which the analyst can define his own analytical work in accordance to the standard procedure.

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1 Scope

This European Standard specifies a method for the determination of vitamin A in foodstuffs by high performance liquid chromatography (HPLC). The determination of vitamin A content is carried out by the measurement of all-trans-retinol, 13-cis-retinol and β-carotene. This part covers the measurement of all-trans-retinol and 13-cis-retinol.

The extract obtained after saponification in this method can be used for the determination of β-carotene, as described in EN 12823-2:1999, Measurement of β-carotene.

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text, and the publications are listed hereafter. For dated references, subsequent amendments to, or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references, the latest edition of the publication referred to applies.

EN ISO 3696 *Water for analytical laboratory use - Specification and test methods* (ISO 3696:1987).

EN ISO 5555 *Animal and vegetable fats and oils - Sampling* (ISO 5555:1991).

3 Principle

Retinol is saponified by using methanolic or ethanolic potassium hydroxide solution and extracted by an appropriate solvent. The determination is carried out by high performance liquid chromatography (HPLC) with either fluorometric (F) or ultra-violet (UV) detection. The substances are identified on the basis of the retention times and determined by the external standard procedure using peak areas or heights, see [1] to [4].

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4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of at least grade 1 according to EN ISO 3696.

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4.1 Methanol

4.2 Ethanol abs., volume fraction, φ (C_2H_5OH) = 100 %.

4.3 Ethanol, φ (C_2H_5OH) = 96 %.

4.4 Sodium sulfate, anhydrous.

4.5 KOH solutions for saponification, in suitable mass concentrations, e.g. ρ (KOH) = 50 g/100 ml or 60 g/100 ml, or alcoholic solutions, e.g. 28 g KOH in 100 ml of an ethanol/water mixture (9+1)(V+V).

4.6 Antioxidants, such as ascorbic acid (AA), sodium ascorbate, sodium sulfide (Na_2S), butylated hydroxytoluene (BHT), pyrogallol or hydroquinone.

4.7 Solvents and extraction solvents such as diethyl ether (peroxide-free), *di*-isopropylether, light petroleum (boiling range of 40 °C to 60 °C), *n*-hexane, butanol or appropriate mixtures thereof.

4.8 HPLC Mobile phases

Examples of appropriate mixtures (expressed as volume parts) include:

n-hexane + 2-propanol (98 + 2);
iso-octane + 2-propanol (98,5 + 1,5);
iso-octane+ *iso*-butanol (98 + 2);
n-hexane + *n*-butanol (98 + 2);
and gradient with 2-propanol + *n*-heptane, (0,5 + 99,5) to (8,5 + 91,5) in 12 min.

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4.9 Standard substances

4.9.1 General

All-trans-retinol (all-trans vitamin A alcohol) and 13-cis-retinol can be obtained in several forms, and from different suppliers. It is therefore necessary to determine the concentration of the calibration solution spectrometrically (see 4.10.4). If vitamin A esters are used (e.g. retinyl palmitate or acetate), check the concentration after saponification (see 7.3.1).

NOTE: Particular attention should be given to the information on the vitamin A content of the standard substances supplied by different manufacturers.

4.9.2 All-trans-retinol, vitamin A alcohol, M ($C_{20}H_{30}O$) = 286,5 g/mol, with a purity of at least 90 %.

4.9.3 Vitamin A esters

4.9.3.1 Retinyl palmitate, vitamin A palmitate, M ($C_{36}H_{60}O_2$) = 524,9 g/mol

4.9.3.2 Retinyl acetate, vitamin A acetate, M ($C_{22}H_{32}O_2$) = 328,5 g/mol, with a purity of at least 90 %.

4.9.4 13-cis-retinol, M ($C_{20}H_{30}O$) = 286,5 g/mol with a purity of at least 60 % for qualitative purposes.

4.10 Stock and standard solutions

4.10.1 All-trans-retinol stock solution

Weigh out approximately 50 mg of all-trans-retinol (4.9.2) to the nearest milligram into a 100 ml one-mark volumetric flask, dissolve in *n*-hexane or other suitable solvents (4.7), and dilute the solution to the mark. The stock solution contains approximately 0,5 mg/ml.

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Alternatively, weigh out approximately 100 mg of retinyl palmitate (4.9.3.1), or 50 mg of retinyl acetate (4.9.3.2) to the nearest milligram into a 100 ml one-mark volumetric flask, and treat as for all-trans-retinol except the concentration should be determined (4.10.4) after saponification (7.3.1).

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Alternative masses and volumes may be used according to chromatographic separation and quantification.

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Store the stock solution protected from light at less than 4 °C.

4.10.2 13-cis-retinol stock solution

Weigh out approximately 1 mg to 2 mg of 13-cis-retinol (4.9.4) to the nearest 0,1 mg into a 100 ml one-mark volumetric flask, dissolve it in absolute ethanol (4.2), or other suitable solvents, and dilute the solution to the mark. This solution contains approximately 10 µg/ml to 20 µg/ml and is used for identification purposes only.

4.10.3 All-trans-retinol standard solution

Pipette 5 ml of the all-trans-retinol stock solution (4.10.1) into a 100 ml one-mark volumetric flask and dilute to the mark with *n*-hexane (4.7) or other suitable solvents compatible with the mobile phase. Pipette 5 ml of this solution into a 50 ml one-mark volumetric flask, and dilute to the mark with the same solvent. The standard solution contains approximately 2,5 µg/ml. Then carry out a concentration and purity test as described in 4.10.4.

Alternatively, retinyl palmiate or retinyl acetate stock solutions (4.10.1) may be used for the preparation of the standard solution. In that case, saponify the solutions using the conditions described in 7.3.1. After extraction and evaporation, redissolve the residue in *n*-hexane or other suitable solvent and proceed as for all-trans-retinol stock solution.

Protect the standard solution from light and store at a temperature of below 4 °C.

4.10.4 Concentration and purity test

Prepare a standard solution of all-trans-retinol in ethanol and measure the absorbance in a quartz cell having an optical path length of 1 cm at the maximum wavelengths of 325 nm to 326 nm with ethanol in the reference cell.

Calculate the mass concentration, $\rho_{\text{all-T}}$, in microgram per millilitre, of all-trans-retinol using equation 1:

$$\rho_{\text{all-T}} = \frac{A_{\text{all-T}} \cdot 10^4}{1830} \cdot P \qquad (1)$$

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Calculate the mass concentration, ρ_{13cis}, in microgram per millilitre, of 13-cis-retinol using equation 2:

$$\rho_{13cis} = \frac{A_{13cis} \cdot 10^4}{1686} \cdot P \qquad (2)$$

where:

$A_{all\text{-}T}$ is the absorption value at the maximum at a wavelength of 325 nm to 326 nm;

A_{13cis} is the absorption value at the maximum at a wavelength of 328 nm;

1830 is the $E_{1cm}^{1\%}$ value for all-trans-retinol solved in ethanol. It may change significantly with other solvents;

1686 is the $E_{1cm}^{1\%}$ value for 13-cis-retinol solved in methanol. It may change significantly with other solvents;

P is the correction factor for purity of all-trans-retinol or 13-cis-retinol assessed by HPLC and calculated using equation 3:

$$P = \frac{B}{B_{total}} \qquad (3)$$

where:

B is the peak area or height for all-trans-retinol or 13-cis-retinol obtained with the standard solution (4.10.3);

B_{total} is the sum of peak areas or heights from all-trans-retinol and 13-cis-retinol obtained with the standard solution (4.10.3).

When using newly purchased vitamin A standard substances, or ones that have been stored for a prolonged period, check whether the absorption maximum of the all-trans-retinol standard solution (4.10.3) used is between 325 nm and 326 nm using a suitable spectrometer.

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For further checks on the vitamin A standards, measure the absorbance of the standard solution in quartz cells (5.1) at wavelengths of 300 nm, 325 nm, 350 nm and 370 nm, with 2-propanol (or other suitable solvents, see 4.7) in the reference path. Determine the following ratio at each wavelength:

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$\frac{E}{E_{325}}$ for all-trans-retinol.

If the ratio does not exceed 0,602 (300 nm), 0,452 (350 nm) and 0,093 (370 nm) for vitamin A alcohol, the standard substance is suitable for use [5], [6].

For retinyl palmitate (4.9.3.1), determine the ratio of E/E_{326} at wavelengths of 300 nm, 350 nm and 370 nm with 2-propanol (or other suitable solvents) in the reference path. If the ratio does not exceed 0,593 (300 nm), 0,537 (350 nm) and 0,142 (370 nm), the standard substance is suitable for use [5], [6], [7].

5 Apparatus

Usual laboratory apparatus and, in particular, the following:

5.1 UV-VIS spectrometer, capable of measuring absorbance at defined wavelengths, with appropriate quartz cells, e.g. of 1 cm path length.

5.2 Rotary evaporator, with water bath and vacuum unit.

NOTE: The use of nitrogen is recommended for releasing of the vacuum.

5.3 HPLC-system, consisting of a high performance liquid chromatograph, pump, sample injection device, a UV-VIS detector or a fluorescence detector and data integrator/manipulation device.

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5.4 HPLC columns

Suitable analytical normal phase columns are appropriate such as LiChrospher® Si 60[1] (5 µm, 250 mm × 4 mm) and LiChrosorb® Si 60[1] (5 µm, 250 mm × 4 mm and 125 mm × 4 mm). The performance criterion for suitable analytical columns is the baseline resolution of all-trans-retinol and 13-cis-retinol.

Column types and particle sizes other than those specified in this European standard may also be used. Chromatographic conditions may have to be adapted for such columns to guarantee equivalent results.

5.5 Filter device

Large and small scale filter devices to filter HPLC mobile phases and sample solutions respectively, e.g. of 0,45 µm pore size is appropriate.

NOTE: Filtering of the mobile phase as well as of the sample test solution through a membrane filter prior to use or injection may increase longevity of the columns.

5.6 Extraction columns (optional), e.g. Extrelut®[1]

5.7 Phase separation filters (optional)

6 Sampling

Sampling shall be in accordance with EN ISO 5555, if appropriate.

7 Procedure

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7.1 General

Vitamin A is sensitive to UV radiation and to oxidizing agents (e.g. atmospheric oxygen). It is therefore necessary to exclude UV light (by using amber glassware, aluminium foil, or UV-absorbing materials) and oxygen (nitrogen flushing) when handling it. In particular, the air above the liquid shall be replaced by a blanket of nitrogen during saponifications. The solvents shall be evaporated under reduced pressure using a rotary evaporator (5.2) at not more than 50 °C.

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7.2 Preparation of the test sample

Homogenize the test sample. Grind coarse material with an appropriate mill and mix thoroughly. Precautions such as pre-cooling shall be taken in order not to expose the sample to high temperatures for long periods of time.

7.3 Preparation of the sample test solution

7.3.1 Saponification

Saponify 2 g to 10 g of the test sample by refluxing preferably under nitrogen using suitable amounts of methanol (4.1) or ethanol (4.3), antioxidants (4.6) (optional) and one of the potassium hydroxide solutions (4.5.1). If antioxidants such as ascorbic acid (AA), pyrogallol or butylated hydroxytoluene are used, they should be added to the sample prior to the addition of the potassium hydroxide solution. Sodium sulfide may also be added to obviate the oxidative catalytic effects of trace metals.

Examples of suitable ratios of reagents are given in Table 1.

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[1] LiChrospher® Si 60, LiChrosorb®Si 60 and Extrelut® are examples of suitable products available commercially. This information is given for the convenience of users of this European Standard, and does not constitute an endorsement by CEN of the products.

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Table 1

Sample mass	Alcohol	Antioxidants	Potassium hydroxide
2 g to 5 g	50 ml methanol	0,25 g AA	5 ml of a 50 g/100 ml solution
5 g to 10 g	100 ml ethanol	1 g AA + 0,04 g Na_2S	20 ml of a 60 g/100 ml solution
10 g to 20 g	150 ml ethanol	1 g AA	50 ml of a 60 g/100 ml solution

The usual time of saponification ranges from 15 min to 45 min with temperatures of 80 °C to 100 °C. Saponification may also be carried out at room temperature overnight (approximately16 h) under otherwise same conditions.

If after saponification and cooling, fat or oil is present on the surface of the saponification mixture, additional ethanolic potassium hydroxide has to be added and saponification time extended.

7.3.2 Extraction

In order to avoid emulsions, an amount of water has to be added to the saponified sample solution so that the ratio of alcohol to water in the resulting solution is 1:1.

Extract the retinol from the saponified sample solution by means of a suitable solvent or a suitable solvent mixture (4.7) and repeat the procedure 3 to 4 times with volumes ranging from 50 ml to 150 ml. Wash the combined extracts to neutral with water (2 to 4 times, 50 ml to 150 ml). The extraction can also be performed by the solid support, liquid/liquid extraction technique (e.g. Extrelut®[2]) when the content of retinol is not too low and the fat content of the sample is less than 10 % [1].

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7.3.3 Evaporation

Evaporate the extract using a rotary evaporator (5.2) under reduced pressure at not more than 50 °C. Remove traces of water by drying with sodium sulfate (4.4), or by azeotropic distillation with absolute ethanol (4.2), or use phase separation filter paper (5.5).

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7.3.4 Dilution

Redissolve the residue preferably using the mobile phase (4.8), or another HPLC compatible solvent in order to obtain an appropriate concentration for injection onto the HPLC column (5.4). This is the sample test solution.

7.4 Preparation of standard solutions

Using standard solutions (4.10.3), prepare calibration solutions for HPLC using suitable solvents to cover the analytical range required.

7.5 Identification and purity check

Identify the all-trans-retinol and 13-cis-retinol by comparing the retention times from sample chromatograms with those obtained from standards under the same chromatographic conditions. Peak identification can also be performed by adding the standard substances to the sample test solution.

NOTE 1: The separation and quantification has proven to be satisfactory if the following chromatographic conditions are followed (also Figure A..1). For alternative HPLC systems, Table C.1.

Stationary Phase:	Merck LiChrosorb® Si60, 5 µm
Column dimension:	250 mm × 4 mm
Mobile phase:	*n*-hexane + *n*-butanol (98 + 2) (volume parts)
Flow:	2 ml/min
Injection volume:	50 µl
Detection:	Fluorescence: Excitation: 325 nm, Emission: 475 nm UV: 325 nm.

[2] See footnote 1).

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