SLOVENSKI STANDARD
SIST EN 12823-2:2000

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Živila - Določevanje vitamina A s tekočinsko kromatografijo visoke ločljivosti - 2. del: Merjenja beta karotena

Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 2: Measurements of Beta-carotene

Lebensmittel - Bestimmung von Vitamin A mit Hochleistungs-Flüssigchromatographie - Teil 2: Bestimmung von ß-Carotin

Produits alimentaires - Dosage de la vitamine A par chromatographie liquide haute performance - Partie 2: Dosage du sz-carotene

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Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 2: Measurement of β-carotene

Produits alimentaires - Dosage de la vitamine A par chromatographie liquide haute performance - Partie 2: Dosage du β-carotène

Lebensmittel - Bestimmung von Vitamin A mit Hochleistungs-Flüssigchromatographie - Teil 2: Bestimmung von β-Carotin

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This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the Central Secretariat has the same status as the official versions.

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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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Contents

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.

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of an identical text or by endorsement, at the latest by August 2000, and conflicting national standards shall be withdrawn at the latest by August 2000.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.

Introduction

As this draft European Standard deals with the measurement of total-β-carotene in foodstuffs, reference is made to the literature for the calculation and expression of β-carotene as vitamin A equivalents [1], [2].

Vitamin A activity can be calculated from the β-carotene data assuming appropriate factors.

1 Scope

This European Standard specifies a method for the determination of total-β-carotene in foodstuffs by high performance liquid chromatography (HPLC).

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).

EN 12823-1:2000 — *Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 1: Measurement of all-trans-retinol and 13-cis-retinol.*

3 Principle

Determination of the sum of β-carotene isomers in an appropriate sample solution by HPLC and spectrometric detection in the visible range. The extract obtained after saponification as described in EN 12823 -1 may be used for quantification. Identification on the basis of the retention times, and determination by the external standard method using peak areas or peak heights, see [3] to [7].

Internal standard methods may also be used if the corresponding recovery tests have proven the same behaviour of the internal standard during the analysis as the analyte itself.

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.

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 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 acetonitrile, diethyl ether (peroxide-free), *di*-isopropylether, light petroleum (boiling range of 40 °C to 60 °C), *n*-hexane, dichloromethane, tetrahydrofuran, toluene or appropriate mixtures thereof.

4.8 Methanolic ammonium acetate solution, e.g. c ($CH_3CO_2NH_4$) = 0,05 mol/l.

4.9 Triethylamine

4.10 HPLC mobile phase, for example acetonitrile (4.7) + methanolic ammonium acetate solution (4.8) + dichloromethane (4.7) (75+20+5) (volume parts) containing 0,1 % by mass of butylated hydroxy toluene (4.6) and 0,05 % by mass of triethylamine (4.9). For mobile phases of alternative HPLC-systems, see annex C.

4.11 Standard substances

4.11.1 General

β-Carotene and α-carotene can be obtained from various suppliers, e.g. Sigma[1]. The purity of the standards can vary between 90 % and 100 %. It is therefore necessary to determine the concentration of the calibration solution spectrometrically (see concentration and purity test [4.12.2]).

4.11.2 β-Carotene, M ($C_{40}H_{56}$) = 536,85 g/mol, with a known mass content of at least 95 %.

4.11.3 α-Carotene, M ($C_{40}H_{56}$) = 536,85 g/mol, for qualitative purposes.

4.11.4 Lycopene, M ($C_{40}H_{56}$) = 536,85 g/mol, for qualitative purposes.

4.12 Stock and standard solutions

4.12.1 β-Carotene stock solution

Dissolve approximately 3 mg, of the β-carotene standard substance (4.11.2) in 20 ml of dichloromethane, tetrahydrofuran or toluene (4.7), placing the volumetric flask for approximately 30 s in an ultrasonic bath (5.6). Dilute this solution with *n*-hexane up to a volume of 100 ml. Dilute 10,0 ml of this solution with *n*-hexane up to 100 ml. 1 ml of this standard solution contains approximately 3 µg β-carotene in *n*-hexane/dichloromethane (98+2) (V/V), *n*-hexane/tetrahydrofuran (98+2) (V/V); or *n*-hexane/toluene (98+2) (V/V).

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

4.12.2 Concentration and purity test

Measure the absorbance of the β-carotene stock solution (4.12.1) at the maximum wavelength of about 453 nm using a spectrometer (5.1). Calculate the mass concentration, ρ, in micrograms per millilitre, using equation (1):

$$\rho = \frac{A_{453} \cdot 10^4}{2592} \qquad (1)$$

where:

A_{453} is the absorption value of the stock solution at the maximum wavelength of about 453 mm;

2592 is the $E^{1\%}_{1cm}$ value of β-carotene in *n*-hexane. It may change considerably with the composition of the solvent [8].

The ratio of A_{455}/A_{340} should be greater than 15, and the ratio A_{455}/A_{483} should be in range 1,14 to 1,18 for pure, all-trans-β-carotene [8].

4.12.3 Standard solution of β-carotene

Pipette 20 ml of the β-carotene stock solution (4.12.1) into a round-bottomed flask and remove the solvent under reduced pressure (5.2) at not more than 50 °C. Dissolve the residue in 20 ml of a solvent compatible to the reversed phase HPLC.

The standard solution shall be stored protected from light and at a temperature below 4 °C and is usually stable for up to 1 week.

4.12.4 Standard solutions of α-carotene and lycopene[2]

For qualitative purposes, pre-dissolve approximately 0,3 mg of α-carotene (4.11.3) or lycopene (4.11.4) in approximately 10 ml of tetrahydrofuran (4.7) and dilute to a volume of 100 ml with ethanol (4.3) or another solvent compatible to the HPLC-system.

The standard solution shall be stored protected from light and at a temperature below 4 °C and is usually stable for up to 1 week.

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1) This information is given for the convenience of users of this standard method and does not constitute an endorsement by CEN of the supplier named. Equivalent products may be used if they can be shown to lead to the same results.

2) The standard solutions of α-carotene and lycopene are not necessary for the quantification of the β-carotene in the sample extract but help to identify clearly the different compounds.

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 the vacuum.

5.3 HPLC-system, consisting of a pump, a sample injecting device, a UV-VIS detector and an evaluation system such as an integrator or recorder.

5.4 HPLC column

Analytical reversed phase column, e.g. C_{18} reversed phase, particle size 5 µm, diameter 4,0 mm to 4,6 mm, length 250 mm.

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

The performance criterion for suitable analytical columns is the resolution factor for all-trans-α-carotene and all-trans-β-carotene which should be greater than 1.

Suitable RP column packing materials are e.g. Vydac® 201TP54[3)], Vydac® 218TP54[3)], Eurospher®100-C_{18}[3)], Ultraspher® ODS[3)], Spherisorb® ODS2[3)], Zorbax® ODS[3)] and LiChrospher® RP 18[3)].The columns may also be used in series. It is also advisable to use a guard column to increase longevity of the analytical column.

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 usually increases longevity of the columns.

5.6 Ultrasonic bath

5.7 Phase separation filter (optional)

6 Sampling

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

7 Procedure

7.1 Preparation of the test sample

Homogenize the test sample. Grind coarse material with an appropriate mill and mix again. Measures such as pre-cooling have to be taken to avoid exposing the sample to high temperatures for long periods of time. β-carotene is sensitive to UV radiation and light.

7.2 Preparation of the sample test solution

7.2.1 Saponification

Saponify 2 g to 10 g of the test sample by refluxing preferably under nitrogen using suitable amounts of ethanol (4.3) or methanol (4.1), water, an antioxidant (4.6) and one of the potassium hydroxide solutions (4.5.1). Add the antioxidants to the sample prior to the addition of the potassium hydroxide. Sodium sulfide (4.6) may also be added to obviate the oxidative catalytic effects of trace metals.

[3)] Vydac® 201TP54, Vydac® 218TP54, Eurospher® 100-C_1 , Ultrasphere® ODS, Spherisorb® ODS2, Zorbax® ODS and LiChrospher® RP18 are available examples of suitable products available commercially. This information is given for the convenience of usersof this standard method and does not constitute an endorsement by CEN of these products.

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

Table 1

Sample mass	Alcohol	Antioxidant	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	1,0 g AA + 0,04 g Na_2S	20 ml of a 60 g/100 ml solution
10 g	150 ml ethanol	1,0 g AA	50 ml of a 60g/100 ml solution

Typical saponification times range from 15 min to 40 min at temperatures of 80 °C to 100 °C (reflux).

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.

NOTE: It has been shown that low fat samples such as fruits or vegetables can be extracted directly with a suitable solvent with a corresponding method e.g. as described in [9] to [11]. However, it is advisable to check that the chromatographic separation fulfils the above defined criteria (problem of interference).

7.2.2 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 β-carotene from the saponified sample solution by means of a suitable solvent or solvent mixture (4.7). Repeat the extraction procedure 3 to 4 times with volumes ranging from 50 ml to 150 ml. Wash the combined extracts to neutral with water (typically 2 to 4 times 50 ml to 150 ml).

7.2.3 Evaporation

Evaporate the extract using a rotary evaporator (5.2) under partial vacuum and at a temperature not exceeding 50 °C. Remove traces of water by drying with sodium sulfate or by azeotropic distillation with abs. ethanol (4.2) or toluene (4.6). Other equivalent techniques such as phase separation filter paper (5.7) to eliminate traces of water may be used provided they have been proven not to affect the result.

7.2.4 Dilution

Re-dissolve the residue in the same solvent mixture in which the standard solutions (4.12.3 and 4.12.4) has been prepared, preferentially the mobile phase or another HPLC-compatible solvent in such a way to obtain a concentration of up to 5 µg/ml of β-carotene. This is the sample test solution.

7.3 Identification

Identify β-carotene by comparison of the retention time of the individual peaks in the chromatograms obtained with the sample test solution (see 7.2.4) and with the standard solution (4.12.3 and 4.12.4). Peak identification can also be performed by adding small amounts of the appropriate standard solution to the sample test solution.

NOTE: The separation and the quantification have proven to be satisfactory if the following chromatographic conditions are followed (see also Figure A.1).

Stationary phase: Spherisorb®[4]ODS2, 5 µm, 100 mm x 4,6 mm cartridge combined with Vydac®[4] 201TP54, 5 µm, 250 mm × 4,6 mm;
Mobile phase: Acetonitrile + methanolic ammonium acetate solution + Dichloromethane (75+20+5) (volume parts) containing 0,1 % by mass of butylated hydroxytoluene and 0,05 % by mass of triethylamine;
Flow rate: 1,5 ml/min;
Injection volume: 50 µl;
Detection: 450 nm.

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[4] Spherisorb® is a product supplied by Phase Separations Inc; Vydac® is a product supplied by The Separations Group. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of this product named. Equivalent products may be used if they can be shown to lead to the same results.

7.4 Determination

Inject appropriate volumes (e.g. 20 µl) of the standard solution (4.12.3) as well as the sample test solution (see 7.2.4) into the HPLC-system (5.3). To carry out a quantitative determination by the external standard method, integrate the peak areas or determine the peak heights obtained for sample test solutions, and compare the results with the corresponding values for the standard substance with similar retention time or construct a calibration curve.

Inject equal volumes of the sample test and of the standard solutions (4.12.3 and 4.12.4) or compensate with a corresponding factor in the calculation of the results. Check the linearity of the calibration function using a minimum of three dilution levels of the β-carotene stock solution (4.12.1).

7.5 Number of determinations

Perform at least two independent determinations.

8 Calculation

Base the calculation on a calibration graph, or use the corresponding programs of the integrator, or use the following simplified procedure.

Calculate the mass concentration, ρ, of total-β-carotene in mg/100 g of the sample using equation (2):

$$\rho = \frac{A_S \cdot c \cdot V_S \cdot V_{St}}{A_{St} \cdot m \cdot V_{iS} \cdot 1000} \cdot 100 \qquad (2)$$

where:

A_S is the peak areas or peak heights for β-carotene-isomers obtained with the sample test solution (see 7.2.4), in units of area or height;
c is the purity corrected (see 4.12.2) concentration of the β-carotene in the standard solution in micrograms per millilitre;
V_S is the total volume of sample test solution (7.2.4) in millilitres;
V_{St} Injection volume of the standard solution, in microlitres;
A_{St} is the peak area or peak height for β-carotene obtained with the standard solution (4.12.3), in units of area or height;
m is the sample mass in grams;
V_{iS} is the injection volume of the sample test solution, in microlitres;
1 000 is the conversion factor (micrograms to milligrams);
100 is the conversion factor for the content per 100 g.

9 Precision

Details of the inter-laboratory test of the precision of the method according to ISO 5725 [12] are summarized in annex B. The value derived from the inter-laboratory test may not be applicable to analyte concentration ranges and matrices other than given in annex B.

9.1 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in not more than 5 % of the cases.

The values for total-β-carotene are:

margarine	$\bar{x}$ = 0,253	mg/100 g	r = 0,032	mg/100 g
vitamin drink	$\bar{x}$ = 2,248	mg/100 g	r = 0,19	mg/100 g
pudding powder	$\bar{x}$ = 1,531	mg/100 g	r = 0,24	mg/100 g
mixed vegetables	$\bar{x}$ = 18,05	mg/100 g	r = 2,0	mg/100 g

9.2 Reproducibility

The absolute difference between two single test results obtained on identical material reported by two laboratories will exceed the reproducibility limit R in not more than 5 % of the cases.