SLOVENSKI STANDARD
SIST EN 14148:2003

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Živila - Določevanje vitamina K1 s HPLC

Foodstuffs - Determination of vitamin K1 by HPLC

Lebensmittel - Bestimmung von Vitamin K1 mit HPLC

Produits alimentaires - Dosage de la vitamine K1 par CLHP

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English version

Foodstuffs - Determination of vitamin K1 by HPLC

Produits alimentaires - Dosage de la vitamine K1 par CLHP

Lebensmittel - Bestimmung von Vitamin K1 mit HPLC

This European Standard was approved by CEN on 2 May 2003.

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

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Contents

Foreword

This document (EN 14148:2003) 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of an identical text or by endorsement, at the latest by January 2004, and conflicting national standards shall be withdrawn at the latest by January 2004.

Annexes A, B and C are informative.

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

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, Hungary, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Slovakia, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Standard specifies a method for the determination of vitamin K_1 in foodstuffs by high performance liquid chromatography (HPLC). The determination of Vitamin K_1 content is carried out by measurement of reduced phylloquinone. The method has been validated for milk and infant formula, however laboratory experiences exist which show that the method is also applicable to other type of foodstuffs [10].

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 (including amendments).

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

3 Principle

After enzymatic removal of fat from the sample vitamin K_1 is determined in an appropriate sample solution by high performance liquid chromatographic separation coupled with post-column reduction and subsequent fluorometric detection. Vitamin K_1 isomers are quantified as a single unresolved peak with a C_{18} column [1] to [4].

4 Reagents

4.1 General

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

4.2 Chemicals and solutions

4.2.1 Methanol, mass fraction $w(CH_3OH) \geq 99{,}8$ %

4.2.2 Ethanol, volume fraction $\varphi(C_2H_5OH) \geq 99{,}8$ %

4.2.3 Reagent alcohol, $\varphi(C_2H_5OH) = 95$ %

Mix 950 ml of ethanol (4.2.2) with 50 ml of methanol (4.2.1).

4.2.4 Dichloromethane, $w(CH_2Cl_2) \geq 99{,}5$ %

4.2.5 n-Hexane, $w(C_6H_{14}) \geq 97$ %

4.2.6 Light petroleum, bp. 35 °C to 60 °C, p.a.

4.2.7 Potassium hydroxide, $w(KOH) \geq 85$ %

4.2.8 Potassium hydroxide solution, substance concentration $c(KOH) = 10$ mol/l

4.2.9 Potassium dihydrogen phosphate, *w*(KH_2PO_4) ≥ 99,5 %

4.2.10 Potassium carbonate, *w*(K_2CO_3) ≥ 99,9 %

4.2.11 Sodium acetate, anhydrous, *w*(CH_3COONa) ≥ 99,5 %

4.2.12 Acetic acid, *w*(CH_3COOH) ≥ 99,8 %

4.2.13 Zinc chloride, *w*($ZnCl_2$) ≥ 98 %

4.2.14 Zinc, powder, particle size < 63 µm, *w*(Zn) ≥ 97 %

4.2.15 Phosphate buffer pH 7,9 to 8,0

Dissolve 54,0 g of potassium dihydrogen phosphate (4.2.9) in approximately 350 ml of water, adjust the pH to 7,9 to 8,0 with potassium hydroxide solution (4.2.8) and dilute to 500 ml with water.

4.2.16 Zinc chloride-acetate solution

Weigh 13,7 g of zinc chloride (4.2.13), 4,1 g of anhydrous sodium acetate (4.2.11) and 3,0 g of acetic acid (4.2.12) in a 50 ml volumetric flask, dissolve in methanol (4.2.1) and dilute to 50 ml with methanol.

4.2.17 Lipase type VII

e.g. from *Candida rugosa*, activity ca. 1000 U/mg or suitable alternative[1)]; other enzyme sources from *Pseudomonas* and *Rhizopus* species can also be used considering the different activity profile.

4.2.18 HPLC Mobile phase

Mix 100 ml of dichloromethane (4.2.4), 900 ml of methanol (4.2.1) and 5 ml of zinc chloride-acetate solution (4.2.16). Filter through a 0,45 µm filter.

4.3 Vitamin K_1 standard substance (Phyllochinone, 3-Phythylmenadione), *w*($C_{31}H_{46}O_2$) ≥ 99%

Vitamin K_1 can be obtained from various suppliers. The purity of the phylloquinone standard may vary. It is therefore necessary to determine the concentration of the calibration solution by UV-spectrometry (see concentration test 4.4.4)

4.4 Stock solutions

4.4.1 Precautions

Vitamin K_1 is very sensitive to light. Measures have to be taken to protect the standard and the corresponding solutions during the whole procedure e.g. by using generally brown glass ware.

4.4.2 Vitamin K_1 stock solution I, mass concentration $\rho(C_{31}H_{46}O_2) \approx 1,0$ mg/ml

Weigh accurately approximately 100 mg of vitamin K_1 standard substance (4.3) into a 100 ml volumetric flask dissolve in methanol (4.2.1) and dilute to 100 ml. This solution can be stored under nitrogen for 3 months at -20 °C in the dark.

NOTE The amount of vitamin K1 can be difficult to dissolve in methanol.

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1) e.g. L-1754; Sigma Chemical Co, P.O. 14508, St. Louis, MO 63178 USA. This product was used in the interlaboratory study. This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.4.3 Vitamin K_1 stock solution II, $\rho(C_{31}H_{46}O_2) \approx 50{,}0$ µg/ml

Pipette 5,0 ml of vitamin K_1 stock solution I (4.4.2), into a 100 ml volumetric flask and dilute to volume with methanol (4.2.1). This solution can be stored under nitrogen for 1 month at –20 °C in the dark.

4.4.4 Concentration test

Evaporate 5,0 ml of vitamin K_1 stock solution II (4.4.3) by means of a rotary evaporator under partial vacuum or under a stream of nitrogen. Redissolve the residue in 25,0 ml of n-hexane (4.2.5) or light petroleum (4.2.6).

Measure the absorbance of this solution in a 1-cm-cell against n-hexane or light petroleum as reference at the maximum wavelength of about 248 nm with a spectrometer (5.1). Calculate the vitamin K_1 mass concentration ρ, in micrograms per millilitre, of the vitamin K_1 stock solution II (4.4.3) according to equation (1):

$$\rho = \frac{A_{248} \times 10^4 \times 5}{419} \qquad (1)$$

where

A_{248} is the absorption value of the solution at the maximum wave length of about 248 nm;

419 is the $A_{1cm}^{1\%}$ value of vitamin K_1 in n-hexane (4.2.5) or light petroleum (4.2.6) at 248 nm[5];

10^4 is the conversion of $A_{1cm}^{1\%}$ to microgramme per milliliter;

5 is the dilution step during solvent change from methanol to n-hexane.

4.5 Standard solutions

4.5.1 Intermediate standard solution, vitamin K_1, ρ ($C_{31}H_{46}O_2$) ≈ 2,5 µg/ml

Pipette 5,0 ml of vitamin K_1 stock solution II (4.4.3), into a 100 ml volumetric flask and dilute to volume with methanol (4.2.1).

4.5.2 Standard test solution for HPLC, vitamin K_1, ρ ($C_{31}H_{46}O_2$) ≈ 25,0 ng/ml

Pipette appropriate volumes e.g. 1 ml of the vitamin K_1 intermediate standard solution (4.5.1) into brown volumetric flasks e.g. 100 ml and add methanol (4.2.1) to dilute to volume. Prepare this solution fresh every day.

5 Apparatus

Use laboratory apparatus and, in particular, the following:

5.1 UV Spectrometer

UV spectrometer capable of measuring absorptions at defined wavelengths, with appropriate cells, e.g. of 1 cm length.

5.2 HPLC system

HPLC system, consisting of a pump, a sample injecting device, a fluorescence detector with an excitation wavelength set at e.g. 243 nm and an emission wavelength set at e.g. 430 nm and an evaluating system such as an integrator.

5.3 HPLC column

Analytical reversed phase column, e.g. of diameter 3,0 mm to 4,6 mm, length 100 mm to 250 mm, filled with particle size 3 µm to 10 µm.

Particle sizes and column dimensions other than those specified in this European Standard may be used. Separation parameters have to be adapted to such materials to guarantee equivalent results.

Other systems (see annex C) can be used providing that a satisfactory separation of phylloquinone from other co-extractives is achieved.

5.4 Post-column reductor

A stainless steel or glass column placed between analytical column and fluorescence detector, e.g. of diameter 2,0 mm to 6,0 mm, length 10 mm to 150 mm, filled with zinc powder (4.2.14).

5.5 Filter device

Membrane filter with pore size of, e.g. 0,45 µm are appropriate.

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

6 Procedure

6.1 Precautions

Vitamin K_1 is very sensitive to light. Measures have to be taken to protect the sample and the corresponding solutions during the whole procedure e.g. by using generally brown glass ware.

6.2 Preparation of the test sample

Homogenise 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 to high temperature for long periods of time.

6.3 Preparation of the sample solution

6.3.1 Sample extraction

Weigh an appropriate amount of the sample to the nearest mg, e.g. 1 g powdery or 10 g liquid material in a closable test tube or a conical flask. Vortex powdery sample with 15 ml of water of 40 °C, supplement liquid samples with 5 ml of water of 40 °C. Run a blank just with the reagents by omitting the sample (see 6.5).

6.3.2 Enzyme treatment

Add 5 ml of phosphate buffer pH 7,9 to 8,0 (4.2.15) and mix. Add 1,0 g lipase (4.2.17), vortex, stopper and shake to disperse for approximately 2 min to 3 min. Incubate the mixture at a temperature of 37 °C ± 2 °C for 2 h. At regular intervals, e.g. 20 min, shake manually the mixture vigorously.

6.3.3 Extraction

Cool to room temperature, add 10 ml of reagent alcohol (4.2.3) and 1,0 g of potassium carbonate (4.2.10) and mix well. Add a defined volume V_E of n-hexane (4.2.5), e.g. 30 ml and shake vigorously. Allow separation of the phases by leaving to stand in the dark or by centrifuging e.g. at 2000 *g* for 10 min. The n-hexane extract can be stored overnight if kept at 4 °C under nitrogen in the dark.