

**SLOVENSKI STANDARD**  
**SIST EN 12823-1:2014****01-julij-2014****Nadomešča:**  
**SIST EN 12823-1:2000**

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**Živila - Določevanje vitamina A s tekočinsko kromatografijo visoke ločljivosti - 1. del: Merjenje vseh trans-retinolov in 13-cis retinola**

Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 1: Measurement 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  
(standards.iteh.ai)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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| 67.050 | Splošne preskusne in analizne metode za živilske proizvode | General methods of tests and analysis for food products |
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EUROPEAN STANDARD

EN 12823-1

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## Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 1: Measurement of all-E-retinol and 13-Z-retinol

Produits alimentaires - Détermination de la teneur en vitamine A par chromatographie liquide haute performance  
- Partie 1: Dosage du tout-E-rétinol et du 13-Z-rétinol

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

This European Standard was approved by CEN on 24 April 2014.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

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

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## Foreword

This document (EN 12823-1:2014) 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 November 2014, and conflicting national standards shall be withdrawn at the latest by November 2014.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 12823-1:2000.

This European Standard consists of two parts:

- *Part 1: Measurement of all-E-retinol and 13-Z-retinol;*
- *Part 2: Measurements of  $\beta$ -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.

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

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## EN 12823-1:2014 (E)

## 1 Scope

This European Standard specifies a method for the determination of vitamin A in foodstuffs by high performance liquid chromatography (HPLC). This method has been validated in an interlaboratory study with samples of margarine and milk powder with all-E-retinol levels ranging from 653 µg/100 g to 729 µg/100 g and with 13-Z-retinol levels ranging from 30 µg/100 g to 39 µg/100 g. The determination of vitamin A content is carried out by the measurement of all-E-retinol, 13-Z-retinol and β-carotene. This part covers the measurement of all-E-retinol and 13-Z-retinol.

The extract obtained after saponification in this method can be used for the determination of β-carotene, as described in EN 12823-2:2000, *Foodstuffs - Determination of vitamin A by high performance liquid chromatography - Part 2: Measurements of β-carotene*. In this case, the saponification temperature should preferably not exceed 80 °C in order to prevent isomerisation and oxidation of β-carotene.

## 2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

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

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## 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 ultraviolet (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].

## 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 absolute**, volume fraction,  $\varphi(\text{C}_2\text{H}_5\text{OH}) = 100 \%$ .

**4.3 Ethanol**,  $\varphi(\text{C}_2\text{H}_5\text{OH}) = 96 \%$ .

**4.4 Sodium sulfate**, anhydrous.

**4.5 KOH solution for saponification**, in suitable mass concentrations, e.g.  $\rho(\text{KOH}) = 50 \text{ g}/100 \text{ ml}$  or  $60 \text{ g}/100 \text{ ml}$ , or alcoholic solutions, e.g. 28 g KOH in 100 ml of a mixture of 9 parts per volume of ethanol and 1 part per volume of water.

**4.6 Antioxidants**, such as ascorbic acid (AA), sodium ascorbate, sodium sulfide ( $\text{Na}_2\text{S}$ ), 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, iso-octane or appropriate mixtures thereof.

## 4.8 HPLC 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.

## 4.9 Standard substances

### 4.9.1 General

All-E-retinol (all-E vitamin A alcohol) and 13-Z-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 6.3.1). Vitamin A and its derivatives are sensitive to oxygen and light. Standard substances should be stored in the dark under nitrogen or argon at  $-20\text{ }^{\circ}\text{C}$ .

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-E-retinol**, vitamin A alcohol,  $M(\text{C}_{20}\text{H}_{30}\text{O}) = 286,5\text{ g/mol}$ , with a purity of at least 90 %.

**4.9.3 Vitamin A esters.**

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**4.9.3.1 Retinyl palmitate**, vitamin A palmitate,  $M(\text{C}_{36}\text{H}_{60}\text{O}_2) = 524,9\text{ g/mol}$ .

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**4.9.3.2 Retinyl acetate**, vitamin A acetate,  $M(\text{C}_{22}\text{H}_{32}\text{O}_2) = 328,5\text{ g/mol}$ , with a purity of at least 90 %.

**4.9.4 13-Z-retinol**,  $M(\text{C}_{20}\text{H}_{30}\text{O}) = 286,5\text{ g/mol}$  with a purity of at least 60 % for qualitative purposes.

## 4.10 Stock and standard solutions

### 4.10.1 All-E-retinol stock solution

Weigh out approximately 50 mg of all-E-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.

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 dilute the solution to the mark. The stock solution concentrations calculated as retinol are approximately 0,55 mg/ml and 0,44 mg/ml, respectively.

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

Store the stock solution protected from light at approximately  $-20\text{ }^{\circ}\text{C}$ . A maximum storage time should be defined based on stability tests carried out by the user under designated conditions.

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## 4.10.2 13-Z-retinol stock solution

Weigh out approximately 1 mg to 2 mg of 13-Z-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-E-retinol standard solution

Pipette 5 ml of the all-E-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 palmitate or retinyl acetate stock solutions (4.10.1) may be used for the preparation of the standard solution. In that case, saponify an aliquot of the stock solution using the conditions described in 6.3.1. After extraction and evaporation, redissolve the residue in *n*-hexane or other suitable solvent and carry out a concentration test as described in 4.10.4.

Protect the standard solution from light and store at a temperature of below 4 °C. A maximum storage time should be defined based on stability tests carried out by the user under designated conditions.

## 4.10.4 Concentration and purity test

Prepare a standard solution of all-E-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,  $\rho_{\text{all-E}}$ , in microgram per millilitre, of all-E-retinol using Formula (1):

$$\rho_{\text{all-E}} = \frac{A_{\text{all-E}} \cdot M_{\text{all-E}} \cdot 10^3}{\varepsilon_{\text{all-E}}} \cdot P \quad \text{SIST EN 12823-1:2014} \quad (1)$$

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Calculate the mass concentration,  $\rho_{13-Z}$ , in microgram per millilitre, of 13-Z-retinol using Formula (2):

$$\rho_{13-Z} = \frac{A_{13-Z} \cdot M_{13-Z} \cdot 10^3}{\varepsilon_{13-Z}} \cdot P \quad (2)$$

where

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

$M_{\text{all-E}}$  is the molar mass (286,5 g/mol) of all-E-retinol;

$\varepsilon_{\text{all-E}}$  is the molar extinction coefficient (52 400) for all-E-retinol dissolved in ethanol, calculated from an  $E_{1\text{cm}}^{1\%}$  value of 1 830 [5], and rounded to 3 significant digits. It may change significantly with other solvents;

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

$M_{13-Z}$  is the molar mass (286,5 g/mol) of 13-Z-retinol;

$\varepsilon_{13-Z}$  is the molar extinction coefficient (48 300) for 13-Z-retinol dissolved in ethanol, calculated from an  $E_{1\text{cm}}^{1\%}$  value of 1 686 [5], and rounded to 3 significant digits. It may change significantly with other solvents;

$P$  is the correction factor for purity of all-E-retinol or 13-Z-retinol assessed by HPLC and calculated using Formula (3):



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

where

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

$B_{\text{total}}$  is the sum of peak areas or heights for all-E-retinol or 13-Z-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-E-retinol standard solution (4.10.3) used is between 325 nm and 326 nm using a suitable spectrometer.

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:

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

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 [6], [7].

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 [6], [7], [8].

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## 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.

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

**5.3 HPLC system**, consisting of a pump, sample injection device, a UV-VIS detector or a fluorescence detector and data integrator/processing device.

### 5.4 HPLC columns

Suitable analytical normal phase columns are appropriate such as LiChrospher<sup>®</sup> Si 60<sup>1)</sup> (5 µm, 250 mm x 4 mm) and LiChrosorb<sup>®</sup> Si 60<sup>1)</sup> (5 µm, 250 mm x 4 mm and 125 mm x 4 mm). The performance criterion for suitable analytical columns is the baseline resolution of all-E-retinol and 13-Z-retinol.

1) LiChrospher<sup>®</sup> Si 60, LiChrosorb<sup>®</sup> Si 60 and Extrelut<sup>®</sup> 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.