SLOVENSKI STANDARD
SIST EN 14122:2014

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Nadomešča:
SIST EN 14122:2003
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Živila - Določevanje vitamina B1 s tekočinsko kromatografijo visoke ločljivosti

Foodstuffs - Determination of vitamin B1 by high performance liquid chromatography

Lebensmittel - Bestimmung von Vitamin B1 mit Hochleistungs-Flüssigchromatographie

Produits alimentaires - Dosage de la vitamine B1 par chromatographie liquide haute performance

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ICS:

67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

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Foodstuffs - Determination of vitamin B1 by high performance liquid chromatography

Produits alimentaires - Détermination de la teneur en vitamine B1 par chromatographie liquide haute performance

Lebensmittel - Bestimmung von Vitamin B1 mit Hochleistungs-Flüssigchromatographie

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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Contents

Page

Foreword

This document (EN 14122:2014) 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of an identical text or by endorsement, at the latest by December 2014 and conflicting national standards shall be withdrawn at the latest by December 2014.

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1 Scope

This European Standard specifies a method for the determination of vitamin B_1 in food by high performance liquid chromatography (HPLC) with enzymatic treatment and pre- or post-column derivatization. This method has been validated in two interlaboratory studies. The first study was for the analysis of samples of whole meal flour, milk powder/spray dried milk, freeze-dried mixed vegetables and freeze-dried pig's liver ranging from 0,295 mg/100 g to 0,807 mg/100 g. The second study was for the analysis of samples of tube feeding solution, baby food with vegetables, powdered milk, meal with fruits, yeast, cereal, chocolate powder and food supplement ranging from 0,11 mg/100 g to 486 mg/100 g. Vitamin B_1 is the mass fraction of total thiamin including its phosphorylated derivatives.

For further information on the validation, see Clause 8 and Annex B.

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)*

3 Principle

Thiamin is extracted from food after acid hydrolysis followed by dephosphorylation using an enzymatic treatment and quantified by HPLC with pre- or post-column derivatization to thiochrome. An external standard is used for quantification. For further information see [1] to [7].

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, or double distilled water.

4.1 Methanol, mass fraction $w(CH_3OH) \geq 99{,}8$ %, HPLC grade.

4.2 Acetic acid solution, substance concentration $c(CH_3COOH) = 0{,}02$ mol/l.

4.3 Isobutanol, $w(C_4H_{10}O) \geq 98$ %.

4.4 Sodium dihydrogen phosphate, $w(NaH_2PO_4) \geq 99{,}8$ %.

4.5 Hydrochloric acid solution, $w(HCl) = 36$ %.

4.6 Hydrochloric acid solution, $c(HCl) = 0{,}1$ mol/l.

4.7 Sulfuric acid solution, $c(H_2SO_4) = 0{,}05$ mol/l.

4.8 Sodium hydroxide, $w(NaOH) \geq 99$ %.

4.9 Sodium hydroxide solution, mass concentration $\rho(NaOH) = 150$ g/l.

4.10 Sodium hydroxide solution, $\rho(NaOH) = 200$ g/l.

4.11 Potassium hexacyanoferrate III, $w\{K_3[Fe(CN)_6]\} \geq 99$ %.

4.12 Potassium hexacyanoferrate III solution, $\rho\{K_3[Fe(CN)_6]\} = 10$ g/l.

4.13 Alkaline potassium hexacyanoferrate III solution (pre-column derivatization), $\rho\{K_3[Fe(CN)_6]\} = 0{,}4$ g/l.

Dilute 2,0 ml of the potassium hexacyanoferrate III solution (4.12) to 50 ml with sodium hydroxide solution (4.9). Prepare fresh each day of analysis.

4.14 Alkaline potassium hexacyanoferrate III solution (post-column derivatization), $\rho\{K_3[Fe(CN)_6]\} = 0{,}5$ g/l.

Dilute 2,5 ml of the potassium hexacyanoferrate III solution (4.12) to 50 ml with sodium hydroxide solution (4.10).

4.15 Enzyme or enzyme mixture, with the ability to liberate vitamin B_1 from foods as free thiamin.

NOTE 1 For the precision data in Table B.1, Taka-Diastase from Pfaltz and Bauer[1)] has been used. For the precision data in Table B.2 and Table B.3 an enzyme mixture of β-amylase from barley and Taka-Diastase from Serva[1)] have been used.

NOTE 2 If incomplete dephosphorylation occurs, this can be solved by the separate quantification of TMP (Thiamin Mono Phosphate), see [7].

4.16 Sodium acetate solution, $c(CH_3COONa \cdot 3H_2O) = 2{,}5$ mol/l.

4.17 Sodium acetate solution, $c(CH_3COONa \cdot 3H_2O) = 0{,}5$ mol/l.

4.18 HPLC mobile phases

Examples of appropriate mixtures with volume fractions of e.g. 10 % to 50 % methanol (4.1) in water or using phosphate or acetate buffer are given in Annex A and Annex C. The possibility of using ion pairing agents is also given.

4.19 Phosphate buffer (pH = 3,5), $c(KH_2PO_4) = 9{,}0$ mmol/l.

4.20 Tetraethylammoniumchloride, $w(C_8H_{20}NCl) \geq 98$ %.

4.21 Sodium heptanesulfonate, $w(C_7H_{15}NaO_3S) \geq 98$ %.

4.22 Acetate buffer (pH = 4,0), $c(CH_3COOH) = 50$ mmol/l.

4.23 Standard substances

4.23.1 Thiamin chloride hydrochloride, $w(C_{12}H_{17}ClN_4OS \cdot HCl) \geq 99$ %.

For external calibration, see 6.3.

4.23.2 Thiamin monophosphate chloride, $w(C_{12}H_{17}ClN_4O_4PS) \geq 98$ %.

For check of enzymes, see 6.2.2.

4.23.3 Thiamin pyrophosphate chloride (cocarboxylase), $w(C_{12}H_{19}ClN_4O_7P_2S) \geq 98$ %.

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1) The information of the suppliers of Taka-Diastase, Pfaltz & Bauer, Waterbury, CT 06708, USA (No T00040), and Serva is given for the convenience of users of this European 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.

For check of enzymes, see 6.2.2.

4.24 Stock solutions

4.24.1 Thiamin chloride hydrochloride stock solution, $\rho(C_{12}H_{17}ClN_4OS \cdot HCl) \approx 0{,}1$ mg/ml.

Dissolve an accurately weighed amount of the thiamin chloride hydrochloride standard substance (4.23.1) in a defined volume of an appropriate solvent, for example 10 mg of vitamin B_1 standard substance in 100 ml of hydrochloric acid solution (4.6). This solution can be stored for four weeks at + 4 °C.

4.24.2 Thiamin monophosphate stock solution, $\rho(C_{12}H_{17}ClN_4O_4PS) \approx 0{,}1$ mg/ml.

Dissolve an accurately weighed amount of the thiamin monophosphate chloride (4.23.2) in a defined volume of an appropriate solvent, for example 10 mg of thiamin monophosphate chloride in 100 ml of hydrochloric acid solution (4.6). This solution can be stored for four weeks at - 20 °C.

4.24.3 Thiamin pyrophosphate stock solution, $\rho(C_{12}H_{19}ClN_4O_7P_2S) \approx 0{,}1$ mg/ml.

Dissolve an accurately weighed amount of the thiamin pyrophosphate chloride (4.23.3) in a defined volume of an appropriate solvent, for example 10 mg of the thiamin pyrophosphate chloride in 100 ml of hydrochloric acid solution (4.6).

4.24.4 Concentration tests - thiamin chloride hydrochloride

Dilute 10 ml of the thiamin chloride hydrochloride solution (4.24.1) with hydrochloric acid solution (4.6) in a 100 ml volumetric flask to the mark. Measure the absorbance of this solution at the maximum of about 247 nm (A_{247}) in a 1 cm cell against hydrochloric solution (4.6) in the reference cell using an UV spectrometer (5.1). Calculate the mass concentration, ρ, in microgram per millilitre thiamin chloride hydrochloride solution (4.24.1) using Formula (1):

$$\rho = \frac{A_{247} \cdot M \cdot 1\,000}{\varepsilon} \qquad (1)$$

where

ε is the molar absorption coefficient of thiamin chloride hydrochloride at the maximum wavelength of about 247 nm. The value is 14 200 l · mol^{-1} · cm^{-1}. This value is calculated from the extinction coefficient, $E_{1cm}^{1\%}$ = 421, in 0,1 mol/l HCl [8][7] and the molar mass, M = 337,21. The value is given with four significant figures;

M is the molar mass, in grams per mol. The value is 337,21;

A_{247} is the absorption value of the thiamin chloride hydrochloride solution.

4.25 Standard solutions

4.25.1 Thiamin chloride hydrochloride standard solution, $\rho(C_{12}H_{17}ClN_4OS \cdot HCl) \approx 1$ µg/ml to 10 µg/ml.

Pipette 1 ml to 10 ml of the thiamin chloride hydrochloride solution (4.24.1) into a 100 ml volumetric flask and dilute to the mark with the appropriate solvent, e.g. hydrochloric acid solution (4.6). This solution can be stored at 4 °C in the dark for 1 month.

4.25.2 Thiamin monophosphate standard solution, $\rho(C_{12}H_{17}ClN_4O_4PS) \approx 1$ µg/ml to 10 µg/ml.

Pipette 1 ml to 10 ml of the thiamin monophosphate solution (4.24.2) into a 100 ml volumetric flask and dilute to the mark with the appropriate solvent, e.g. hydrochloric acid solution (4.6). This solution can be stored at 4 °C in the dark for 1 month.

4.25.3 Thiamin pyrophosphate standard solution, $\rho(C_{12}H_{19}ClN_4O_7P_2S) \approx 1$ µg/ml to 10 µg/ml.

Pipette 1 ml to 10 ml of the thiamin pyrophosphate solution (4.24.3) into a 100 ml volumetric flask and dilute to the mark with the appropriate solvent, e.g. hydrochloric acid solution (4.6). This solution can be stored at 4 °C in the dark for 1 month.

5 Apparatus

Usual laboratory apparatus, glassware, and the following:

5.1 UV spectrometer, UV spectrometer, capable of measuring absorption at defined wavelengths (247 nm), with appropriate cells, e.g. of 1 cm length.

5.2 Autoclave or heating device, autoclave for extraction purpose, e.g. pressure cooker type, with pressure or temperature reading device, electrical heating device or water bath.

5.3 HPLC system

HPLC system, consisting of a pump, a sample injecting device, a fluorescence detector with an excitation and emission wavelength set at e.g. 366 nm and 435 nm, respectively (see Annex C), and an evaluation system such as an integrator.

5.4 HPLC column

5.4.1 General

Other particle sizes or column dimensions than those specified in this European Standard may be used. Separation parameters shall be adapted to such materials to guarantee equivalent results. The performance criterion for suitable analytical columns is the baseline resolution of the thiamin from interferences[2)].

5.4.2 Pre-column oxidation

Analytical columns, e.g. Lichrospher® 60 RP Select B [2)], particle size of 5 µm, diameter 4,0 mm to 4,6 mm, length 100 mm to 250 mm.

5.4.3 Post-column oxidation

Analytical columns, e.g. Supelco® LC-18- DB [2)], particle size of 5 µm, diameter 4,0 mm to 4,6 mm, length 100 mm to 250 mm.

5.5 Filter device

Filtering of the mobile phase as well as of the sample solution through a membrane filter with, e.g. a pore size of 0,45 µm, prior to use or injection will increase longevity of the columns.

5.6 Post-column reactor pump and derivatization tube, a suitable reagent delivery system, a T-type connecting tube and a derivatization tube (e.g. 10 m x 0,33 mm).

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2) Suitable silica column packing materials available commercially are Lichrosorb® Si 60, Spherisorb® Si, Hypersil® Si and Lichrospher® 100 DIOL. Suitable RP column packing materials are Spherisorb® ODS, µ-Bondapak® radial C18, Supelco® LC-18- DB and Hypersil® ODS. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of these products.

6 Procedure

6.1 Preparation of the test sample

Homogenize the test sample. Grind coarse material with an appropriate mill and mix again. Measures such as pre-cooling shall be taken to avoid exposing to high temperature for long periods of time.

6.2 Preparation of the sample test solution

6.2.1 Extraction

Weigh an appropriate amount of the test sample to the nearest mg, e.g. 2 g to 10 g in a conical flask. Add a defined volume ranging from 60 ml to 200 ml of hydrochloric acid solution (4.6), or sulfuric acid solution (4.7). The pH of the solution should not be higher than pH = 2,0. Cover the container with a watch glass and either autoclave the test portion at 121 °C for 30 min, or heat it at 100 °C for 60 min.

The data from the BCR study have shown that a wide range of conditions for the acid hydrolysis can be applied (temperature 95 °C to 130 °C, time 15 min to 60 min). The higher the temperature is, the shorter the time should be.

6.2.2 Enzyme treatment

After cooling to room temperature adjust the extract to the optimal pH for the enzyme used with sodium acetate solution (4.16) or (4.17) and add a suitable amount of enzyme or enzyme mixture (4.15) to the sample. Incubate the mixture at the optimal time and temperature for the enzyme(s) used. After cooling to room temperature transfer the solution to a volumetric flask using distilled water, or another appropriate solvent and dilute the sample test solution to a defined volume (V_{ts}).

For each enzyme used, optimal pH, incubation time and incubation temperature shall be checked.

To ensure an optimal dephosphorylation, the enzymatic step shall be checked with analysis of samples spiked with thiamin monophosphate chloride (4.23.2) or thiamin pyrophosphate chloride (4.23.3), and a material similar in sample type as the test sample. This material should be a certified reference material.

The amount of thiamin possibly brought in with the enzyme(s) (4.15) shall be considered in the calculation of the result.

NOTE For determination of the precision data given in this European Standard in Table B.1, Table B.2 and Table B.3, Taka-Diastase and Taka-Diastase combined with β-amylase from barley was used for dephosphorylation under the following conditions. The extract was adjusted to pH = 4,0 and pH = 4,5, respectively, with sodium acetate solution (4.16) or (4.17) and 100 mg of Taka-Diastase and 10 mg β-amylase per gram of sample was added. The mixture was incubated at 37 °C to 45 °C for 4 h to 24 h, see [5], [10] and [16].

6.2.3 Sample test solution

If necessary, filter the sample solution (6.2.2) through a filter paper or a 0,45 µm membrane filter. Centrifugation may also be used. This is the sample test solution for oxidation (6.3.2 or 6.3.3).

6.3 Oxidation of thiamin to thiochrome

6.3.1 General

The oxidation may be performed either pre-column (6.3.2) or post-column (6.3.3).

6.3.2 Pre-column oxidation

6.3.2.1 Procedure for oxidation step

Pipette 1 ml of the enzymatically treated sample (6.2.3), standard (4.25.1) or blank i.e. hydrochloric acid solution (4.6) or sulfuric acid solution (4.7) depending which was used in 6.2.1 into suitable vials or flasks, add 1 ml of alkaline hexacyanoferrate III solution (4.13). Shake the sample test solution for a fixed amount of time (e.g. 10 s), leave to stand for a specified time (e.g. 1 min).

In order to remove interfering compounds and to protect the HPLC column it is recommended to neutralize the sample test solution (e.g. with H_3PO_4) or to perform a clean-up using solid phase extraction (for more information see [5]).

Filter through a 0,45 µm membrane filter. This is the sample test solution to inject into the reverse phase HPLC system (6.3.2.2).

Alternatively, the oxidized solution can be extracted into 1,5 ml isobutanol (4.3) and the extract can be injected.

NOTE The oxidative conversion of thiamin to thiochrome can be inhibited in some foods. This phenomena is often encountered in cocoa containing foods, but can also be observed in other foods. If such a problem is suspected, it is recommended to check the recovery of the method by spiking the sample extract with an appropriate volume of thiamin standard solution before the oxidation reaction.

6.3.2.2 Identification with HPLC after pre-column oxidation

Inject the same appropriate volumes of the solutions (6.3.2.1) of standards, samples and blank into the HPLC system. Identify the thiochrome by comparison of the retention time of the individual peaks in the chromatograms obtained with the sample test solution, and with the standard test solution. Adding the standard substances to the sample test solution can also perform peak identification.

The separation and the quantification were proven to be satisfactory if the following experimental conditions are followed (see Annex C and Figure A.1 for alternative HPLC conditions and examples of chromatograms).

Stationary phase:	Lichrospher® RP Select B, 5 µm, 250 mm x 4,0 mm
Mobile phase:	40 parts per volume of methanol (4.1) and 60 parts per volume of acetate buffer (4.22)
Flow rate:	0,7 ml/min
Injection volume:	20 µl
Detection:	Fluorometric: excitation: 366 nm and emission: 435 nm

6.3.3 Post-column oxidation

6.3.3.1 Procedure for oxidation step

Oxidize thiamin to thiochrome using a post-column reaction with the alkaline potassium hexacyanoferrate III solution (4.14). Add continuously (e.g. 0,3 ml/min) the derivatization reagent through a T-type connecting tube to the HPLC eluent to form the thiochrome.

NOTE The post-column oxidation step is influenced e.g. by the sodium hydroxide concentration. Higher concentrations in the derivatization solution can be compensated by a lower pump rate and vice versa.

6.3.3.2 Identification with HPLC using post-column oxidation

Inject the same appropriate volumes of the standards of thiamin chloride hydrochloride (4.23.1) as well as of the sample solutions (6.2.3) into the HPLC system. Identify the thiamin by comparison of the retention time of the