SLOVENSKI STANDARD
SIST EN 14152:2003

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Živila - Določevanje vitamina B2 s HPLC

Foodstuffs - Determination of vitamin B2 by HPLC

Lebensmittel - Bestimmung von Vitamin B2 mit HPLC

Produits alimentaires - Détermination de la teneur en vitamine B2 par CLHP

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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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Foodstuffs - Determination of vitamin B2 by HPLC

Produits alimentaires - Dosage de la vitamine B2 par CLHP

Lebensmittel - Bestimmung von Vitamin B2 mit HPLC

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

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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 Management Centre has the same status as the official versions.

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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 14152:2003) 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of an identical text or by endorsement, at the latest by January 2004, and conflicting national standards shall be withdrawn at the latest by January 2004.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 may 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 B_2 in foodstuffs by high performance liquid chromatography (HPLC). The determination of vitamin B_2 content is carried out by measurement of riboflavin.

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

Riboflavin in an appropriate sample solution is determined after acid hydrolysis followed by dephosphorylation using an enzymatic treatment by high performance liquid chromatographic (HPLC) separation with a fluorometric detection [1] to [8].

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 double distilled water.

4.2 Chemicals and solutions

4.2.1 Methanol, mass fraction, $w(CH_3OH) \geq 99,8$ % HPLC grade

4.2.2 Sodium acetate trihydrate, $w(CH_3COONa \cdot 3H_2O) = 99$ %

4.2.3 Sodium acetate solution, substance concentration $c(CH_3COONa \cdot 3H_2O) = 0,1$ mol/l

4.2.4 Sodium acetate solution, $c(CH_3COONa \cdot 3H_2O) = 2,5$ mol/l

4.2.5 Glacial acetic acid, $w(CH_3COOH) = 99,8$ %

4.2.6 Acetic acid solution, $c(CH_3COOH) = 0,02$ mol/l

4.2.7 Hydrochloric acid, $w(HCl) = 36$ %

4.2.8 Hydrochloric acid, $c(HCl) = 0,1$ mol/l

4.2.9 Hydrochloric acid, $c(HCl) = 0,01$ mol/l

4.2.10 Sulfuric acid, $c(H_2SO_4) = 0,05$ mol/l

4.2.11 Sodium hydroxide, $w(NaOH) = 99$ %

4.2.12 Sodium hydroxide solution, $c(NaOH) = 0,5$ mol/l

4.2.13 Phosphorous pentoxide, $w(P_2O_5) = 98$ %

4.2.14 Dephosphorylating enzyme, with the ability to hydrolyse bound riboflavin from food[1)]

4.2.15 HPLC Mobile phase

Examples of appropriate mixtures of e. g. 10 % to 50 % methanol (4.2.1) in water or using phosphate or acetate buffer are given in annex A1 and C; possibility of using ion-pairing agents is also given.

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1) e.g. Taka-Diastase Nr. T00040, Pfalz & Bauer, Waterbury, CT 06708, USA was used for the collaborative study. This information is given for the convenience of users of this standard and does not constitute endorsement by CEN of the product named. Equivalent products may be used if they can be shown to lead to the same results [7, 10, 11].

4.2.16 Phosphate buffer (pH 3,5), $c(KH_2PO_4)$ = 9,0 mmol/l

4.2.17 Tetraethylammoniumchloride, $w(C_8H_{20}NCl)$ = 98 %

4.2.18 Sodium heptanesulfonate, $w(C_7H_{15}NaO_3S)$ = 98 %

4.3 Standard substances

4.3.1 Riboflavin, $w(C_{17}H_{20}N_4O_6)$ = 98 %;

Vitamin B_2 can be obtained as riboflavin from various suppliers. The purity of the riboflavin standard may vary. It is therefore necessary to determine the concentration of the calibration solution by UV-spectrometry (see concentration test 4.4.3).

4.3.2 Riboflavin-5'-phosphate

Riboflavin-5'-phosphate sodium salt, $w(C_{17}H_{20}N_4NaO_9P)$ = 95 %; for qualitative purposes only.

4.4 Stock solution

4.4.1 Precautions

Vitamin B_2 is very sensitive to light. Measures have to be taken to protect the vitamin B_2 and the corresponding solutions during the whole sample preparation procedure e. g. by using generally brown glassware.

4.4.2 Riboflavin standard stock solution, mass concentration $\rho(C_{17}H_{20}N_4O_6) \approx 100$ µg/ml

Dissolve an amount of riboflavin standard substance (4.3.1) previously dried and stored in dark in a desiccator possibly under vacuum or/and over phosphorous pentoxide (4.2.13), weighed to the nearest milligram, e. g. approximately 50 mg in a defined volume, e. g. 500 ml in an appropriate solvent e. g. diluted acetic acid (4.2.6) using brown volumetric flasks. This solution can be stored at 4 °C in the dark for 2 months.

Riboflavin is sparingly soluble. To facilitate dissolution warm with ca. 300 ml diluted acetic acid (4.2.6), on a steam bath with constant stirring until dissolved, cool and add diluted acetic acid (4.2.6) to make 500 ml. Alternatively add 5 ml sodium hydroxide solution (4.2.12) to the standard substance in a 500 ml volumetric flask. Due to the instability in alkaline solutions immediately after dissolution add 1,5 ml of glacial acetic acid (4.2.5) and dilute to volume with diluted acetic acid (4.2.6), or another appropriate acid. The concentration of the freshly prepared and if necessary also stored solution should be tested (4.4.3).

4.4.3 Concentration test

Mix 20 ml of the riboflavin stock solution, (4.4.2) in a 200 ml volumetric flask with 3,5 ml sodium acetate solution (4.2.3) and dilute with water to the mark. For the preparation of the blind solution, mix 20 ml of acetic solution (4.2.6) with 3,5 ml of sodium acetate solution in a 200 ml volumetric flask and dilute to the mark with water. Take these solutions for the spectrometric measurement.

Measure the absorbance of the riboflavin solution at the maximum wavelength of about 444 nm in a 1 cm cell with a spectrometer (5.1) against the blind solution as reference. Calculate the mass concentration, ρ, of riboflavin in micrograms per millilitre, of the stock solution (4.4.2) according to equation (1):

$$\rho = \frac{A_{444} \cdot 10^4 \cdot 10}{328} \qquad (1)$$

where

A_{444} is the absorption value of the solution at the maximum wavelength of about 444 nm;

328 is the $E_{1cm}^{1\%}$ value of riboflavin (in acetate buffer, pH 3,8) at 444 nm [9];

10 is the dilution factor.

4.5 Standard solutions

4.5.1 Standard solution, $\rho(C_{17}H_{20}N_4O_6) \approx 10$ µg/ml

Prepare a 1:10 dilution of the riboflavin stock solution (4.4.2), e. g. pipette 10 ml of the riboflavin stock solution (4.4.2), into a 100 ml brown volumetric flask and add diluted acetic acid (4.2.6) or another appropriate solvent to make 100 ml. Prepare this solution fresh every day.

4.5.2 Standard test solutions, $\rho(C_{17}H_{20}N_4O_6) \approx 0{,}1$ µg/ml to 1 µg/ml

Pipette corresponding volumes e. g. 1,0 ml to 10,0 ml of the standard solution (4.5.1), into brown volumetric flasks e. g. 100 ml and dilute with the mobile phase (4.2.15) to the mark. 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 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 wavelength set at e. g. 468 nm and an emission wavelength set at e. g. 520 nm and an evaluating system such as an integrator.

5.4 HPLC column

Analytical reversed phase column, e. g. of diameter 4,0 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 riboflavin from other co-extractives is achieved.

5.5 Filter device

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

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

6 Procedure

6.1 Precautions

Vitamin B_2 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 glassware.

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 test solution

6.3.1 Sample extraction

Weigh an appropriate amount of the sample to the nearest mg, e. g. 2 g to 10 g in a beaker or a conical flask. The defined volume ranging from 50 ml to 200 ml of hydrochloric acid (4.2.8), or sulfuric acid (4.2.10). The pH of the solution should be less than 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.

NOTE 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, the shorter the time). However, prolonged heating of riboflavin and riboflavin-5'-phosphate may cause losses. It has been shown that, notably for chocolate foods, the extraction value could drop when pH was above 2.

6.3.2 Enzyme treatment

After cooling to room temperature adjust the extract to pH 4,0 with sodium acetate solution (4.2.4) and add 100 mg Taka-Diastase (4.2.14) per gram of sample. Incubate the mixture at 37 °C to 46 °C for 16 h to 24 h. After cooling to room temperature transfer to a light protected volumetric flask using diluted acetic acid (4.2.6) or an other appropriate solvent and dilute to a defined volume (V_E).

NOTE The dephosphorylation dependens on the sample matrix and on the enzyme (mixture) used [7], [10], [11].

To insure an optimal dephosphorylation the enzymatic step should be checked with an appropriate sample known to contain corresponding vitamin B_2 phosphates or by addition of riboflavin-5'-phosphate (4.3.2) to a test sample and observing the adequate conversion to riboflavin. The conversion is sufficient if riboflavin-5'-phosphate is not shown in the chromatogram.

The enzyme used for the dephosphorylation, Taka-Diastase (4.2.14), may contain riboflavin. The amount of riboflavin brought in with the enzyme has to be considered in the calculation of the result.

6.3.3 Sample test solution

If necessary, dilute an aliquot (V_a) from a clear filtrate to a defined volume of the sample solution through a filter paper or use a 0,45 µm membrane filter. Centrifugation at appropriate g level may also be used to clarify the sample solution. From the clear filtrate dilute an aliquot (V_A) to a defined volume (V) with an appropriate solvent mixture to be compatible with the HPLC system used, or e. g. dilute 1,0 ml of the extract (6.3.2) with 1,0 ml of methanol (4.2.1). This is the sample test solution for the HPLC analysis.