SLOVENSKI STANDARD
SIST ENV 14164:2002

01-junij-2002

þ]j]`U!`8 c`c  Yj Ub^Y`j]HUa]bU6 * `g`< D@7

Foodstuffs - Determination of vitamin B6 by HPLC

Lebensmittel - Bestimmung von Vitamin B6 mit HPLC

Produits alimentaires - Détermination de la vitamine B6 par CLHP

Ta slovenski standard je istoveten z: ENV 14164:2002

iTeh STANDARD PREVIEW
(standards.iteh.ai)
SIST ENV 14164:2002
https://standards.iteh.ai/catalog/standards/sist/7b3215e7-a5e4-4337-bc74-d41eb858dcff/sist-env-14164-2002

ICS:

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

SIST ENV 14164:2002 **en**

2003-01.Slovenski inštitut za standardizacijo. Razmnoževanje celote ali delov tega standarda ni dovoljeno.

iTeh STANDARD PREVIEW
(standards.iteh.ai)

SIST ENV 14164:2002
https://standards.iteh.ai/catalog/standards/sist/7b3215e7-a5e4-4337-bc74-d41eb858dcff/sist-env-14164-2002

SIST ENV 14164:2002

EUROPEAN PRESTANDARD

PRÉNORME EUROPÉENNE

EUROPÄISCHE VORNORM

ENV 14164

February 2002

ICS 67.050

English version

Foodstuffs - Determination of vitamin B6 by HPLC

Produits alimentaires - Détermination de la vitamine B6 par CLHP

Lebensmittel - Bestimmung von Vitamin B6 mit HPLC

This European Prestandard (ENV) was approved by CEN on 15 November 2001 as a prospective standard for provisional application.

The period of validity of this ENV is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the ENV can be converted into a European Standard.

CEN members are required to announce the existence of this ENV in the same way as for an EN and to make the ENV available promptly at national level in an appropriate form. It is permissible to keep conflicting national standards in force (in parallel to the ENV) until the final decision about the possible conversion of the ENV into an EN is reached.

CEN members are the national standards bodies of Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and United Kingdom.

iTeh STANDARD PREVIEW
(standards.iteh.ai)
SIST ENV 14164:2002
https://standards.iteh.ai/catalog/standards/sist/7b3215e7-a5e4-4337-bc74-d41eb858dcff/sist-env-14164-2002

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

Management Centre: rue de Stassart, 36 B-1050 Brussels

© 2002 CEN All rights of exploitation in any form and by any means reserved worldwide for CEN national Members.

Ref. No. ENV 14164:2002 E

SIST ENV 14164:2002

Foreword

This European Prestandard has been prepared by Technical Committee CEN/TC 275, "Food analysis - Horizontal methods", the secretariat of which is held by DIN.

The annexes A, B, C and D are informative.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to announce this European Prestandard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Prestandard specifies a method for the determination of vitamin B_6 in foodstuffs by HPLC.

Vitamin B_6 is the mass fraction of the sum of pyridoxine, pyridoxal, pyridoxamine including their phosphorylated derivatives determined as pyridoxine. The β-glycosylated forms are not taken into account.

iTeh STANDARD PREVIEW
(standards.iteh.ai)

2 Normative references

This European Prestandard 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 Prestandard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

SIST ENV 14164:2002
https://standards.iteh.ai/catalog/standards/sist/7b3215e7-e5e4-4327-be74-d41eb858cbff/sist-env-14164-2002

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

3 Principle

Pyridoxal, pyridoxamine and pyridoxine are extracted from food by acid hydrolysis and dephosphorylated enzymatically using acid phosphatase.

By reaction with glyoxylic acid in presence of Fe++ as catalyst, pyridoxamine is transformed into pyridoxal, which is then reduced to pyridoxine by the action of sodium borohydride in alkaline medium. Pyridoxine is then quantified in the sample solution by HPLC with a fluorometric detection [1], [2].

It is also possible to quantify pyridoxine, pyridoxal and pyridoxamine separately and to sum the different contents obtained. In that case all risk of interference with the matrix will have to be verified and the analytical conditions adapted. The procedure is not described in this European Prestandard because it has not been validated. Adapted conditions can be found in the bibliography [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 double distilled water.

SIST ENV 14164:2002

4.2 Chemicals and solutions

4.2.1 Acid phosphatase, from potatoes (it can be obtained from Boehringer)[1] Enzymatic activity: 2 U/mg.

4.2.2 Sodium acetate, trihydrate, w ($CH_3COONa \cdot 3\ H_2O$) ≥ 99,0 %

4.2.3 Glacial acetic acid, w (CH_3COOH) ≥ 99,8 %

4.2.4 Glyoxylic acid, w ($C_2H_2O_3 \cdot H_2O$) ≥ 97,0 %

4.2.5 Ferrous sulfate II, heptahydrate, w ($FeSO_4 \cdot 7H_2O$) ≥ 99,5 %

4.2.6 Sodium hydroxide, w ($NaOH$) ≥ 98,0 %

4.2.7 Sodium borohydride, w ($NaBH_4$) ≥ 97,0 %

4.2.8 Potassium dihydrogen phosphate, w (KH_2PO_4) ≥ 99,0 %

4.2.9 Orthophosphoric acid, w (H_3PO_4) ≥ 84,0 %

4.2.10 Sodium octanesulfonate, w ($C_8H_{17}NaO_3S$) ≥ 98,0 %, or sodium heptanesulfonate, w ($C_7H_{15}NaO_3S$) ≥ 98,0 %

iTeh STANDARD PREVIEW
(standards.iteh.ai)

4.2.11 Acetonitrile (HPLC grade), w (C_2H_3N) ≥ 99,8 %

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

SIST ENV 14164:2002
https://standards.iteh.ai/catalog/standards/sist/7b3215e7-a5e4-4337-bc74-d41eb858def1/sist-env-14164-2002

Dissolve 170,1 g of sodium acetate, trihydrate (4.2.2) in 500 ml of water.

4.2.13 Sodium acetate solution, c ($CH_3COONa \cdot 3H_2O$) = 0,05 mol/l (pH 4,5):

Dissolve 6,8 g of sodium acetate, trihydrate (4.2.2) in 1 l of water. Adjust the pH to 4,5 with glacial acetic acid (4.2.3).

4.2.14 Ferrous sulfate solution, c ($FeSO_4 \cdot 7H_2O$) = 0,0132 mol/l

Dissolve 36,6 mg of ferrous sulfate II, heptahydrate (4.2.5) in 10 ml of sodium acetate solution (4.2.13).

4.2.15 Sodium hydroxide solution, c ($NaOH$) = 0,2 mol/l

Dissolve 800 mg of sodium hydroxide (4.2.6) in 100 ml of water.

4.2.16 Sodium borohydride solution, c ($NaBH_4$) = 0,1 mol/l

Dissolve 378 mg of sodium borohydride (4.2.7) in 100 ml of sodium hydroxide solution (4.2.15).

4.2.17 Glyoxylic acid solution, c ($C_2H_2O_3 \cdot H_2O$) = 1 mol/l (pH 4.5):

Dissolve 4,7 g of glyoxylic acid monohydrate (4.2.4) in 30 ml of sodium acetate solution (4.2.12). Adjust the pH to 4,5 with the sodium acetate solution (4.2.12) and dilute to 50 ml with water in a volumetric flask.

---

1) This information is given for the convenience of users of this standard method 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.

SIST ENV 14164:2002

4.2.18 Hydrochloric acid, *c* (HCl) = 0,1 mol/l

4.2.19 HPLC mobile phase

In a beaker add 940 ml of water, 40 ml of acetonitrile (4.2.11), 160 mg of sodium octanesulfonate or sodium heptanesulfonate (4.2.10) and 6,8 g of potassium dihydrogen phosphate (4.2.8).

After shaking, adjust the pH to 2,5 with orthophosphoric acid (4.2.9). Transfer the solution in a 1 l volumetric flask. Adjust to the mark with water.

Filter through a 0,45 µm filter.

4.3 Vitamin B_6 standard substance

Pyridoxine hydrochloride, w ($C_8H_{11}NO_3 \cdot HCl$) ≥ 99 %

4.4 Stock solution

4.4.1 Vitamin B_6 stock solution, ρ ($C_8H_{11}NO_3 \cdot HCl$) ≈ 0,5 mg/ml

Dissolve an accurately weighed amount of the vitamin B_6 standard substance (4.3), e.g. approximately 50 mg in a defined volume, e.g. 100 ml, of water. The stock solution is stable for 4 weeks if stored at 4 °C in the dark.

4.4.2 Concentration test

iTeh STANDARD PREVIEW
(standards.iteh.ai)

Dilute 0,5 ml of vitamin B_6 stock solution (4.4.1) to 20 ml with 0,1 mol/l HCl (4.2.18) and measure the absorbance at 290 nm in a 1 cm cell using a UV-spectrometer (5.2) against 0,1 mol/l HCl solution as reference.

Calculate the mass concentration ρ, in microgram per millilitre of the stock solution according to equation (1):

SIST ENV 14164:2002
https://standards.iteh.ai/catalog/standards/sist/7b3215e7-a5e4-4337-bc74-d41eb858dcff/sist-env-14164-2002

$$\rho = \frac{A \cdot M_w}{8,6} \cdot F \qquad (1)$$

where

- A is the absorbance value of the solution at 290 nm;
- M_w is the molecular weight of vitamin B_6 standard substance, in gram per mol;
- F is the dilution factor, i.e. 40.
- *8,6* is the molar extinction coefficient ε of pyridoxine hydrochloride in 0,1 mol/l hydrochloric acid at 290 nm [4], [5], in $mmol^{-1}$ cm^{-1};

Further information on molar extinction coefficients in other solutions than 0,1 mol/l HCl (pH ~ 1) can be seen in annex D.

4.5 Standard solutions

4.5.1 Vitamin B_6 intermediate standard solution, ρ ($C_8H_{11}NO_3 \cdot HCl$) ≈ 10 µg/ml

Pipette 1 ml of the vitamin B_6 stock solution (4.4.1) into a 50 ml volumetric flask and dilute to the mark with water.

Prepare this solution each day of analysis.

SIST ENV 14164:2002

4.5.2 Vitamin B_6 standard test solutions for HPLC

$\rho(C_8H_{11}NO_3 \cdot HCl)$~0,1µg/ml to 1µg/ml

Prepare a series of appropriate standard solutions of concentrations ranging from e.g. 0,1 µg/ml to 1 µg/ml of pyridoxine hydrochloride by using the pyridoxine intermediate standard solution (4.5.1).

Prepare these solutions each day of analysis.

5 Apparatus

5.1 General

Usual laboratory apparatus, glassware, and, the following:

5.2 UV Spectrometer

Capable of measurement of absorbance at defined wavelengths.

5.3 Heating device

Oven or water bath, with shaking facilities.

iTeh STANDARD PREVIEW

5.4 High performance liquid chromatographic system

(standards.iteh.ai)

Consisting of a pump, sample injecting device, fluorescence detector with excitation and emission wavelengths set at 290 nm and 395 nm, respectively and an evaluation system such as an integrator.

SIST ENV 14164:2002
https://standards.iteh.ai/catalog/standards/sist/7b3215e7-a5e4-4337-bc74-d41e6b98dea/sist-env-14164-2002

5.5 HPLC-Column, e.g. reversed phase column, such as LiChrospher® 60 RP C8 Select B[2)], particle size of 5 µm, diameter 4,0 mm, length 250 mm.

Other particle sizes or column dimensions than specified in this European Prestandard may be used. Separation parameters have to be adapted to such materials to guarantee equivalent results. The performance criterion for suitable analytical columns is the baseline resolution of the analytes concerned.

5.6 Filter device

Filtering of the mobile phase as well as of the test 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.

6 Procedure

6.1 Preparation of the test sample

Homogenise the test sample. Grind coarse material with an appropriate mill and mix again. Measures such as precooling have to be taken to avoid exposing to high temperature for long periods of time.

2) LiChrospher ® 60 RP C8 Select B is an example of a suitable product available commercially. This information is given for the convenience of users and does not constitute an endorsement by CEN of the product named. Equivalent products can be used if they lead to equivalent results.

SIST ENV 14164:2002

6.2 Preparation of the sample test solution

6.2.1 Extraction

Weigh an appropriate amount of the sample to the nearest milligram, e.g. 2,5 g (if the vitamin B_6 content exceeds 2 µg/g) or 5 g (if the vitamin content is less than 2 µg/g) in a conical flask. Add 50 ml of hydrochloric acid (4.2.18). Heat for 30 min in a boiling water bath.

NOTE 1 For samples, with a high water content or low contents of vitamin B_6, it can be useful to increase the sample weight, e.g. 20 g, to add an adapted volume of water, e.g. 25 ml, and to add directly 5 ml HCl 1 mol/l.

NOTE 2 For samples with a high fat content it can be useful to remove fat with e.g. light petroleum before the acid hydrolysis.

NOTE 3 The existing data in annex B have been mainly obtained without any acid hydrolysis. The main advantage is to improve the filtration step for samples with starch. A modification of the procedure (without acid hydrolysis) is described in annex C.

6.2.2 Enzyme treatment and transformation steps

After cooling to room temperature, adjust the extract to pH 4,5 with sodium acetate solution (4.2.12) and add 2,5 ml of 1 mol/l glyoxylic acid (4.2.17), 400 µl of ferrous sulfate solution (4.2.14) and 20 mg of acid phosphatase (4.2.1). Incubate the solution overnight at 37 °C with continuous agitating.

After cooling to room temperature, dilute to 100 ml with water in a volumetric flask. Shake and filter. Add a 5 ml aliquot to 4,5 ml of 0,1 mol/l sodium borohydride solution (4.2.16). Shake at least for 3 min. In order to assure the complete destruction of the excess of sodium borohydride, it is possible to add 0,5 ml of glacial acetic acid (4.2.3). Take care for the dilution factor. Shake for 1 min. Filter through 0,45 µm filter. Use this filtrate for chromatography.

iTeh STANDARD PREVIEW
(standards.iteh.ai)

6.2.3 Identification

SIST ENV 14164:2002

Identify the pyridoxine by comparison of the retention time of the individual peaks in the chromatograms obtained with the test sample solution, and with the standard test solution. Peak identification can also be performed by adding the pyridoxine standard substance to the sample test solution.

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

Stationary phase:	LiChrospher ® 60 RP C8 Select B, 5 µm, 250 mm x 4,0 mm;
Mobile phase:	according to 4.2.19;
Flow rate:	1 ml/min;
Injection volume:	30 µl;
Detection:	Fluorometric: Excitation: 290 nm; Emission: 395 nm.

6.2.4 Determination by HPLC

Inject the same appropriate volumes (up to 50 µl) of the standard solution as well as of the sample test solution into the HPLC-system. To carry out a determination by external calibration, integrate the peak areas or peak heights and compare the results with the corresponding values for the standard substance.

7 Calculation

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

SIST ENV 14164:2002

Calculate the mass fraction, w, of vitamin B_6 as pyridoxine in mg/100 g of the sample using equation (2):

$$w = \frac{A_S \cdot \rho \cdot 100 \cdot 2}{A_{St} \cdot m \cdot 1000} \cdot 100 \cdot 0{,}822 \qquad (2)$$

where

A_S is the peak area or peak height for pyridoxine obtained with the sample test solution, in units of area or height;

A_{St} is the peak area or peak height for pyridoxine obtained with the standard test solution, in units of area or height;

ρ is the mass concentration of pyridoxine hydrochloride in the standard test solution, in micrograms per millilitre;

m is the sample mass, in gram;

100 is the total volume of the sample test solution, in millilitres;

2 is the factor of dilution of the reaction with sodium borohydride;

1 000 is the factor to convert microgram to milligram;

100 is the factor to calculate the content per 100 g;

0,822 is the factor to convert pyridoxine hydrochloride to pyridoxine.

iTeh STANDARD PREVIEW
(standards.iteh.ai)

Report the result for vitamin B_6 calculated as pyridoxine in mg/100g.

SIST ENV 14164:2002
https://standards.iteh.ai/catalog/standards/sist/7b3215e7-a5e4-4337-bc74-d41eb858dcff/sist-env-14164-2002

8 Test report

The test report shall contain at least the following data:

a) all information necessary for the complete identification of the sample;

b) a reference to this European Prestandard or to the method used;

c) date and type of sample procedure (if known);

d) date of sample receipt;

e) date of test;

f) the results and the units in which the results have been expressed;

g) any particular points observed in the course of the test;

h) any operations not specified in the method or regarded as optional which might have affected the results.