SLOVENSKI STANDARD
SIST EN 14164:2014

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Nadomešča:
SIST EN 14164:2008

Živila - Določevanje vitamina B6 s tekočinsko kromatografijo visoke ločljivosti

Foodstuffs - Determination of vitamin B6 by high performance chromatography

Lebensmittel - Bestimmung von Vitamin B6 mit Hochleistungs-Flüssigchromatographie

Produits alimentaires - Dosage de la vitamine B6 par chromatographie liquide haute performance

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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 B6 by high performance chromatography

Produits alimentaires - Détermination de la teneur en vitamine B6 par chromatographie liquide haute performance

Lebensmittel - Bestimmung von Vitamin B6 mit Hochleistungs-Flüssigchromatographie

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Foreword

This document (EN 14164:2014) 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of an identical text or by endorsement, at the latest by December 2014 and conflicting national standards shall be withdrawn at the latest by December 2014.

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This document supersedes EN 14164:2008.

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

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

1 Scope

This European Standard specifies a method for the determination of vitamin B_6 in foodstuffs by high performance liquid chromatography (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. These can be determined with the method given in EN 14663 [1] by which the different vitamins of vitamin B_6 (pyridoxal, pyridoxamine and pyridoxine) are separated and individually quantified. A third European Standard, EN 14166 [2], determines the total vitamin B_6 by microbiological assay.

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

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

By reaction with glyoxylic acid in the presence of Fe^{2+} as a 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 [3], [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, or double distilled water.

4.1 Acid phosphatase, (CAS 9001-77-8), from potatoes, enzyme activity is 33 nkat/mg[1)] with substrate p-nitrophenyl phosphate at pH = 4,8 and T = 37 °C, for example from Boehringer or Sigma[2)]. 33 nkat/mg corresponds to 2 U/mg.

4.1.1 Acid phosphatase solution

Prepare a solution of 20 mg/ml acid phosphatase in sodium acetate solution (4.14).

It is necessary to use acid phosphatase rather than Taka-diastase to obtain a complete hydrolysis of phosphorylated forms of vitamin B_6. Where 300 mg of Taka-diastase is needed to obtain good dephosphorylation, only 0,5 mg of acid phosphatase is needed, see [5].

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1) Katal (symbol "kat") is a derived SI unit of enzyme activity. One katal is that catalytic activity which will raise the rate of reaction by one mol/s in a specified assay system.

2) This information is given for the convenience of users of this European standard and does not constitute an endorsement by CEN of the supplier. Equivalent products may be used if they can be shown to lead to the same results.

4.1.2 Activity check of acid phosphatase

The activity of acid phosphatase can be checked as proposed below.

Prepare a stock solution of approximately 0,1 mg/ml of pyridoxal phosphate (4.9) in water.

Mix 3,0 ml of the PLP stock solution and 10 ml of hydrochloric acid (4.21) in a 20 ml volumetric flask and fill up to the mark with water. Check the concentration of PLP by measuring the absorbance at 293 nm in a 1 cm cell using a UV-spectrometer (5.1) against a hydrochloric acid solution (4.20) as reference. The molar absorption coefficient (ε) of PLP in 0,1 mol/l HCl is 7 200.

Calculate the mass concentration ρ_{PLP} of the stock solution, in milligram per millilitre, according to Formula (1):

$$\rho_{PLP} = \frac{A_{293} \cdot M_{PLP}}{\varepsilon} \cdot F \quad (1)$$

where

A_{293} is the absorption value of the solution at 293 nm;

M_{PLP} is the molar mass of vitamin B_6 standard substance, in gram per mol (M_{PLP} = 247,14);

F is the dilution factor (here F = 20/3);

ε is the molar absorption coefficient of PLP in 0,1 mol/l of hydrochloric acid at 293 nm, in l mol^{-1} cm^{-1}, (here: ε = 7 200), see [6].

Take 1,0 ml of the PLP stock solution for extraction and proceed with 6.2.1, 6.2.2, 6.2.3 and 6.2.4.

Calculate the pyridoxine (PN) conversion rate from the dephosphorylated pyridoxal phosphate solution according to Formula (2):

$$\text{Conversion rate}\,(\%) = \frac{\rho_{PN \cdot HCl} \cdot A_S \cdot 2 \cdot 100 \cdot 0{,}822 \cdot 100 \cdot M_{PLP}}{A_{ST} \cdot 1\,000 \cdot \rho_{PLP} \cdot M_{PN}} \quad (2)$$

where

$\rho_{PN\ HCL}$ is the mass concentration of pyridoxine hydrochloride in the standard test solution, in micrograms per millilitre;

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

2 is the factor of dilution of the reaction with sodium borohydride if acetic acid is added, otherwise the dilution factor is 1,9;

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

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

100 is the conversion factor for %;

M_{PLP} is the molar mass of pyridoxal phosphate (PLP), in gram per mol (M_{PLP} = 247,14);

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

1 000 is the factor to convert microgram to milligram;

ρ_{PLP} is the mass concentration of pyridoxal phosphate (PLP) in the stock solution, in milligrams per millilitre;

M_{PN} is the molar mass of pyridoxine (PN), in gram per mol (M_{PN} = 169,1).

4.2 Sodium acetate, trihydrate, mass fraction $w(CH_3COONa \cdot 3H_2O) \geq 99,0$ %.

4.3 Glacial acetic acid, $w(CH_3COOH) \geq 99,8$ %.

4.4 Glyoxylic acid, $w(C_2H_2O_3 \cdot H_2O) \geq 97,0$ %.

4.5 Ferrous sulfate II, heptahydrate, $w(FeSO_4 \cdot 7H_2O) \geq 99,5$ %.

4.6 Sodium hydroxide, $w(NaOH) \geq 99,0$ %.

4.7 Sodium borohydride, $w(NaBH_4) \geq 97,0$ %.

4.8 Potassium dihydrogen phosphate, $w(KH_2PO_4) \geq 99,0$ %.

4.9 Pyridoxal phosphate (PLP), $w \geq 99,0$ %.

4.10 Orthophosphoric acid, $w(H_3PO_4) \geq 84,0$ %.

4.11 Sodium octanesulfonate, $w(C_8H_{17}NaO_3S) \geq 98,0$ %, or sodium heptanesulfonate, $w\ (C_7H_{15}NaO_3S) \geq 98,0$ %.

4.12 Acetonitrile (HPLC grade), $w(C_2H_3N) \geq 99,8$ %.

4.13 Sodium acetate solution, substance concentration $c(CH_3COONa \cdot 3H_2O) = 2,5$ mol/l.

Dissolve 170,1 g of sodium acetate, trihydrate (4.2) in 500 ml of water.

4.14 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) in 1 l of water. Adjust the pH to 4,5 with glacial acetic acid (4.3).

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

Dissolve 36,6 mg of ferrous sulfate II, heptahydrate (4.5) in 10 ml of sodium acetate solution (4.14). Prepare fresh each day of use.

NOTE In a study described by Mann et al., see [7], a ferrous sulfate solution of 10 g/l was used. This concentration was based on the completion of the conversion of pyridoxamine to pyridoxal at pyridoxamine levels up to 8 times the minimum level of vitamin B_6 required by the infant formula Act in the US, see Mann et al. [8]. This concentration seems not to be necessary for the European situation.

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

Dissolve 800 mg of sodium hydroxide (4.6) in 100 ml of water.

4.17 Sodium hydroxide solution, $c(NaOH) = 6,0$ mol/l.

Dissolve 24 g of sodium hydroxide (4.6) in 100 ml of water.

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

Dissolve 378 mg of sodium borohydride (4.7) in 100 ml of sodium hydroxide solution (4.16). Prepare fresh on day of use.

4.19 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 (4.4) in 30 ml of sodium acetate solution (4.13). Adjust the pH to 4,5 with the sodium hydroxide solution (4.17) and dilute to 50 ml with water in a volumetric flask. Prepare fresh on day of use.

4.20 Hydrochloric acid, c(HCl) = 0,1 mol/l.

4.21 Hydrochloric acid, c(HCl) = 0,2 mol/l.

4.22 HPLC mobile phase

In a beaker add 940 ml of water, 40 ml of acetonitrile (4.12), 160 mg of sodium octanesulfonate or sodium heptanesulfonate (4.11) and 6,8 g of potassium dihydrogen phosphate (4.8).

After dissolving sodium octanesulfonate or sodium heptanesulfonate and potassium dihydrogen phosphate by stirring, adjust the pH to 2,5 with orthophosphoric acid (4.10). Transfer the solution in a 1 l volumetric flask. Adjust to the mark with water. Filter through a 0,45 µm filter.

4.23 Pyridoxine hydrochloride (vitamin B_6 standard substance), $w(C_8H_{11}NO_3 \cdot HCl) \geq 99$ %.

4.24 Pyridoxine hydrochloride stock solution, mass concentration $\rho \approx 0,5$ mg/ml.

Dissolve an accurately weighed amount of pyridoxine hydrochloride (4.23), e.g. 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.

For the concentration test, dilute 0,5 ml of pyridoxine hydrochloride stock solution (4.24) to 20 ml with 0,1 mol/l HCl (4.20) and measure the absorbance at 290 nm in a 1 cm cell using a UV-spectrometer (5.1) against 0,1 mol/l HCl solution as reference. Calculate the mass concentration ρ, in microgram per millilitre of the stock solution according to Formula (3):

$$\rho_{PNHCl} = \frac{A_{290} \cdot M_{PNHCl} \cdot 1\,000}{\varepsilon} F \quad (3)$$

where

A_{290} is the absorption of the value of the solution at 290 nm;

M_{PNHCl} is the molar mass of vitamin B_6 standard substance, in gram per mol (M_{PNHCl} = 205,64);

F is the dilution factor (here F = 40);

ε is the molar absorption coefficient of pyridoxine hydrochloride in 0,1 mol/l of hydrochloric acid at 291 nm, in l mol^{-1} cm^{-1} (here ε = 8 600), see [9].

Further information on molar absorption coefficients in other solutions than 0,1 mol/l HCl (pH ≈ 1) is given in Annex D.

4.25 Standard solutions

4.25.1 Pyridoxine hydrochloride intermediate standard solution, $\rho(C_8H_{11}NO_3 \cdot HCl) \approx 10$ µg/ml.

Pipette 1 ml of the vitamin B_6 stock solution (4.24) into a 50 ml volumetric flask and dilute to the mark with water. Prepare this solution each day of analysis.

4.25.2 Pyridoxine hydrochloride standard test solution for HPLC, $\rho(C_8H_{11}NO_3 \cdot HCl) \approx 0,1$ µg/ml to 1 µg/ml.