SLOVENSKI STANDARD
SIST EN 14164:2008

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Nadomeġļa:
SIST ENV 14164:2002

Ģivila - Doloļevanje vitamina B6 s HPLC

Foodstuffs - Determination of vitamin B6 by HPLC

Lebensmittel - Bestimmung von Vitamin B6 mit HPLC

Produits alimentaires - Dosage de la vitamine B6 par CLHP

Ta slovenski standard je istoveten z: EN 14164:2008

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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 B_6 by HPLC

Produits alimentaires - Dosage de la vitamine B_6 par CLHP

Lebensmittel - Bestimmung von Vitamin B_6 mit HPLC

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
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Foreword

This document (EN 14164:2008) 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of an identical text or by endorsement, at the latest by December 2008, and conflicting national standards shall be withdrawn at the latest by December 2008.

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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 vitamers of vitamin B_6 (pyridoxal, pyridoxamine and pyridoxine) are separated and individually quantified. A third European Standard (EN 14166 [1]) [2] determines the total vitamin B_6 by microbiological assay.

2 Normative references

The following referenced document is indispensable for the application of this document. 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 3696:1987).*

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 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

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 Acid phosphatase, (CAS 9001-77-8), from potatoes, enzyme activity is 33 nkat/mg [2)] with substrate p-nitrophenyl phosphate at pH = 4,8 and T = 37 °C, for example from Boehringer or Sigma [3)]. 33 nkat/mg corresponds to 2 U/mg.

4.2.1.1 Acid phosphatase solution

Prepare a solution of 20 mg/ml acid phosphatase in sodium acetate solution (4.2.14).

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[1] Under elaboration.

2) 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.

3) 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.

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].

4.2.1.2 Activity check of acid phosphatase

Check the activity of each new batch of acid phosphatase as follows. Prepare a stock solution of approximately 0,1 mg/ml of pyridoxal phosphate (4.2.9) in water. Take 5,0 ml of this solution for extraction and procede with 6.2.1, 6.2.2, 6.2.3, 6.2.4 and 7.

Calculate the pyridoxine recovery from this solution and divide by the theoretical amount of pyridoxine released from pyridoxal phosphate (PLP). Calculate the theoretical mass concentration ρ_{PN} of PN released from PLP, in milligram per millilitre according to equation (1):

$$\rho_{PN} = \frac{\rho_{PLP\ UV} \times M_{PN} \times 2 \times 5{,}0}{100 \times M_{PLP}} \qquad (1)$$

where

$\rho_{PLP\ UV}$ is the mass concentration of PLP determined by UV spectrometry;

M_{PN} is the molecular weight of vitamin B_6 standard substance, in gram per mol (M_{PN} = 169.1);

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

5,0 is the volume taken for extraction, see 4.2.1.2;

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

M_{PLP} is the molecular weight of PLP, in gram per mol (M_{PLP} = 265,16).

Mix 3,0 ml of the PLP stock solution and 10 ml of hydrochloric acid (4.2.21) in a 20 ml volumetric flask and fill up to the mark with water. Check the concentration of PLP by measuring the absorbance at 295 nm in a 1 cm cell using a UV-spectrometer (5.2) against a hydrochloric acid solution (4.2.20) as reference. Molar absorption coefficient (ε) of PLP in 0,1 mol/l HCl is 8 353.

Calculate the mass concentration ρ_{PLP} of the stock solution, in milligram per millilitre, according to equation (2):

$$\rho_{PLP} = \frac{A_{295} \times M_{PLP}}{\varepsilon} \times F \qquad (2)$$

where

A_{295} is the absorption of the value of the solution at 295 nm;

M_{PLP} is the molecular weight of vitamin B_6 standard substance, in gram per mol (M_{PLP} = 265,16);

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 295 nm, in l mol^{-1} cm^{-1} (here ε = 8 353).

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

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

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

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

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

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

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

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

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

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

$w(C_7H_{15}NaO_3S) \geq 98,0$ %

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

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

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

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

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

4.2.15 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 (4.2.14). Prepare fresh each day of use.

NOTE In a study described by Mann et al., see [10], 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. [9]. This concentration seems not to be necessary for the European situation.

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

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

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

Dissolve 24 g of sodium hydroxide (4.2.6) in 100 ml of water.

4.2.18 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 (4.2.16). Prepare fresh on day of use.

4.2.19 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 (4.2.4) in 30 ml of sodium acetate solution (4.2.13). Adjust the pH to 4,5 with the sodium hydroxide solution (4.2.17) and dilute to 50 ml with water in a volumetric flask. Prepare fresh on day of use.

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

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

4.2.22 HPLC mobile phase

In a beaker add 940 ml of water, 40 ml of acetonitrile (4.2.12), 160 mg of sodium octanesulfonate or sodium heptanesulfonate (4.2.11) and 6,8 g of potassium dihydrogen phosphate (4.2.8).

After dissolving sodium octanesulfonate or sodium heptanesulfonate and potassium dihydrogen phosphate by stirring, adjust the pH to 2,5 with orthophosphoric acid (4.2.10). Transfer the solution in a 1 l volumetric flask. Adjust to the mark with water. Filter through a 0,45 µm filter.

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

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

Dissolve an accurately weighed amount of pyridoxine hydrochloride (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.

For the concentration test, dilute 0,5 ml of pyridoxine hydrochloride stock solution (4.4) to 20 ml with 0,1 mol/l HCl (4.2.20) 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 (3):

$$\rho_{PNHCl} = \frac{A_{290} \times M_{PNHCl} \times 1000}{\varepsilon} \times F \qquad (3)$$

where

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

M_{PN} is the molecular weight of vitamin B_6 standard substance, in gram per mol (M_{PN} = 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 [6] .

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