SLOVENSKI STANDARD
SIST EN 14663:2006

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Živila - Določevanje vitamina B6 (vključno z glikosilirano obliko) s HPLC

Foodstuffs - Determination of vitamin B6 (including its glycosylated forms) by HPLC

Lebensmittel - Bestimmung von Vitamin B6 (einschließlich glucosidisch gebundener Verbindungen) mit HPLC

Produits alimentaires - Dosage de la vitamine B6 (y compris ses formes glycosylées) par CLHP

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Foodstuffs - Determination of vitamin B6 (including its glycosylated forms) by HPLC

Produits alimentaires - Dosage de la vitamine B6 (y compris ses formes glycosylées) par CLHP

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Foreword

This document (EN 14663:2005) 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of an identical text or by endorsement, at the latest by June 2006, and conflicting national standards shall be withdrawn at the latest by June 2006.

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1 Scope

This document 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 as well as the β-glycosylated forms, calculated as pyridoxine.

This method has been successfully validated with semolina with milk (infant food), potato puree, vegetables with ham (convenient products) and a multi vitamin drink at levels from 0,034 mg/100 g to 1,21 mg/100 g.

2 Normative references

The following referenced documents are 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 and deglycosilated enzymatically using acid phosphatase and β-glucosidase.

The different derivatives of vitamin B_6 (pyridoxal, pyridoxamine and pyridoxine) are separated by HPLC and quantified by fluorometric detection [1], [2].

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 Di-potassium hydrogen phosphate, mass fraction $w(K_2HPO_4 \cdot 3\ H_2O) \geq 99{,}9\ \%$

4.3 Sodium acetate, without crystal water, $w(CH_3COONa) \geq 99{,}0\ \%$

4.4 Trichloroacetic acid (TCA), $w(Cl_3CCOOH) \geq 99{,}0\ \%$

4.5 Sodium acetate solution, substance concentration $c(CH_3COONa) = 2{,}5$ mol/l

Dissolve 205 g of sodium acetate (4.3) in 1 l of water.

4.6 Post-column reagent (optional), K_2HPO_4 solution $c(K_2HPO_4) = 0{,}15$ mol/l

Dissolve 34,2 g of di-potassium hydrogen phosphate (4.2) in water, dilute to 1 000 ml, mix and degas.

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

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

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

4.10 Sulfuric acid, $c(H_2SO_4)$= 1 mol/l

4.11 Light petroleum, boiling range of 40 °C to 60 °C

4.12 Acid phosphatase, from potatoes. Enzymatic activity approximately 5,3 U/mg[1)].

It is important that the enzyme used complies with the activity check 4.13.2, for further information see [2], [7].

4.13 Acid phosphatase solution

4.13.1 General

Dissolve/solubilise 60 mg of acidic phosphatase (4.12) in 10 ml of water in a beaker by stirring for 2 min. Prepare this solution on the day of analysis.

4.13.2 Activity check of Acid Phosphatase

Weigh 10 g of pork, 5 g of potato puree or 5 g of whole meal into a beaker, and extract with acid as described in 6.2.1. Add 1 ml of acid phosphatase solution (4.13.1) and optional 1 ml of β-glucosidase solution (4.15) to 12,5 ml of the extracted sample solution and mix. Incubate the solution at least 12 h or overnight at 37 °C with continuous stirring. Repeat this step with the double amount of acid phosphatase solution.

Determine the mass concentration of vitamins according to 6.6. The activity of the enzyme used is sufficient, if the resulting mass concentrations of vitamin B_6 compounds in both sample solutions are equivalent. The chromatogram shall not show a peak arising from pyridoxamin phosphate.

NOTE For the interlaboratory test, the acid phosphatase from Sigma Nr P 3752[2)] has been used.

4.14 β-Glucosidase, from almonds. Enzymatic activity of approximately 3,2 U/mg.

It is important that the enzyme used complies with the activity check 4.15.2, for further information see [2], [7].

4.15 β-Glucosidase solution

4.15.1 General

Dissolve/solubilise 100 mg of β-glucosidase (4.14) in 10 ml of water in a beaker by stirring for 2 min. Prepare this solution on the day of analysis.

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1) U, this unit (often called the International unit or standard unit) is defined as the amount of enzyme which catalyses the transformation of 1 µmol substrate per minute under standard conditions.

2) This information is given for the convenience of users of this document 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.

4.15.2 Activity check of β-glucosidase

Weigh 10 g of pork, 5 g of potato puree or 5 g of whole meal into a beaker, and extract with acid as described in 6.2.1. Add 1 ml of acid phosphatase solution (4.13.1) and 1 ml of β-glucosidase solution (4.15.1) to 12,5 ml of the extracted sample solution and mix. Incubate the solution at least 12 h or overnight at 37 °C with continuous stirring. Repeat this step with the double amount β-glucosidase solution.

Determine the mass concentration of vitamin B_6 compounds according to 6.6. The activity of the enzyme used is sufficient, if the resulting mass concentrations of vitamin B_6 compounds in both sample solutions are equivalent. The chromatogram shall not show a peak arising from pyridoxamin phosphate.

NOTE For the interlaboratory test, the β-glucosidase from Sigma Nr G-0395 [1)] has been used.

4.16 Mobile phase for HPLC (Sulfuric acid, $c(H_2SO_4)$= 0,015 mol/l containing 0,005 mol/l TCA)

Dissolve 817 mg ± 5 mg of trichloroacetic acid (4.4) in 15 ml of 1 mol/l sulfuric acid (4.10), transfer into a 1 000 ml volumetric flask, dilute to the mark with water, mix and degas.

4.17 Silicon oil, for defoaming

4.18 Standard substances

4.18.1 General

Pyridoxamine (PM), Pyridoxal (PL) and pyridoxine (PN) can be obtained from various suppliers. The purity of the standards may vary, and it is therefore necessary to determine the concentration and purity (see 4.19.4 and 4.20.7).

4.18.2 Pyridoxamine (PM) dihydrochloride, $w(C_8H_{12}N_2O_2 \cdot 2HCl) \geq 98$ %

4.18.3 Pyridoxal (PL) hydrochloride, $w(C_8H_9NO_3 \cdot HCl) \geq 98$ %

4.18.4 Pyridoxine (PN) hydrochloride, $w(C_8H_{11}NO_3 \cdot HCl) \geq 98$ %

4.19 Stock solutions

4.19.1 Pyridoxamine (PM) stock solution, mass concentration ρ(PM) approximately 500 µg/ml

Dissolve 71,7 mg of pyridoxamine dihydrochloride (4.18.2) in a 100 ml volumetric flask in 0,1 mol/l HCl (4.8) and dilute to the mark with 0,1 mol/l HCl. The solution can be stored without any losses for up to one week at 4 °C or up to two months at -18 °C.

4.19.2 Pyridoxal (PL) stock solution, ρ(PL) approximately 500 µg/ml

Dissolve 60,9 mg of pyridoxal hydrochloride (4.18.3) in a 100 ml volumetric flask in 0,1 mol/l HCl (4.8) and dilute to the mark with 0,1 mol/l HCl. The solution can be stored without any losses for up to one week at 4 °C or up to two months at -18 °C.

[1)] This information is given for the convenience of users of this document 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.

4.19.3 Pyridoxine (PN) stock solution, ρ(PN) approximately 500 µg/ml

Dissolve 60,8 mg of pyridoxine hydrochloride (4.18.4) in a 100 ml volumetric flask in 0,1 mol/l HCl (4.8) and dilute to the mark with 0,1 mol/l HCl. The solution can be stored without any losses for up to one week at 4 °C or up to two months at – 18 °C.

4.19.4 Concentration tests

Pipette 1 ml of stock solutions of pyridoxamine (4.19.1), pyridoxal (4.19.2) and pyridoxine (4.19.3) respectively in a 50 ml volumetric flask and dilute to the mark with 0,1 mol/l HCl (4.8). Measure the absorbance of the solutions in a 1 cm quartz-cell against 0,1 mol/l HCl at the maximum wavelength using UV-spectrometry (see Table 1).

Calculate the mass concentration of each vitamin B_6 compound, ρ_i, using the molar extinction coefficient as given in equation (1):

$$\rho_i = \frac{A \times M_i}{\varepsilon_i} \times V \times F \qquad (1)$$

where:

ρ_i is the mass concentration of pyridoxamine, pyridoxal and pyridoxine respectively in microgram per millilitre stock solution;

A is the absorbance value of pyridoxamine, pyridoxal and pyridoxine solutions at the maximum wavelength λ_{max} (see table 1);

ε_i is the molecular absorbance coefficient of PM, PL or PN at the appropriate pH as defined in table 1;

M_i is the molecular weight of PM, PL and PN respectively standard substances as defined in table 1;

V is the dilution factor, in this case V = 50;

F is the calculation factor of HCl free vitamin B_6 compounds.

Use these mass concentrations to calculate the exact concentrations of 4.19.1 to 4.19.3 and 4.20.1 to 4.20.6.

Table 1 — Examples for molecular extinction coefficients of vitamin B_6 compounds

Compounds	Solvent	λ_{max}	ε_i mmol^{-1}cm^{-1}	M_i g mol^{-1}	F
PM · 2 HCl[a]	0,1 mol/l HCl, pH ~1	292	8,2	241,1	0,698
PL · HCl[b]	0,1 mol/l HCl, pH ~1	288	9,0	203,6	0,821
PN · HCl[c]	0,1 mol/l HCl, pH ~1	291	8,6	205,6	0,823
[a] PM · 2 HCl = Pyridoxamine-dihydrochloride (4.18.2)					
[b] PL · HCl = Pyridoxal-hydrochloride (4.18.3)					
[c] PN · HCl = Pyridoxine-hydrochloride (4.18.4)					

4.20 Standard solutions

4.20.1 Pyridoxamine (PM) standard solution I, ρ(PM) approximately 10 µg/ml

Dilute 2 ml of pyridoxamine stock solution (4.19.1) with 0,1 mol/l HCl (4.8) to 100 ml. Prepare freshly every day

4.20.2 Pyridoxal (PL) standard solution I, ρ(PL) approximately 10 µg/ml

Dilute 2 ml of PL stock solution (4.19.2) with 0,1 mol/l HCl (4.8) to 100 ml. Prepare freshly every day

4.20.3 Pyridoxine (PN) standard solution I, ρ(PN) approximately 10 µg/ml

Dilute 2 ml of pyridoxine stock solution (4.19.3) with 0,1 mol/l HCl (4.8) to 100 ml. Prepare freshly every day

4.20.4 Pyridoxamine (PM) standard solution II, ρ(PM) approximately 1 µg/ml

Dilute 10 ml of standard solution I (4.20.1) with 0,1 mol/l HCl (4.8) to 100 ml. Prepare freshly every day

4.20.5 Pyridoxal (PL) standard solution II, ρ(PL) approximately 1 µg/ml

Dilute 10 ml of PL standard solution I (4.20.2) with 0,1 mol/l HCl (4.8) to 100 ml. Prepare freshly every day

4.20.6 Pyridoxine (PN)standard solution II, ρ(PN) approximately 1 µg/ml

Dilute 10 ml of pyridoxine standard solution I (4.20.3) with 0,1 mol/l HCl (4.8) to 100 ml. Prepare freshly every day

4.20.7 Check of chromatographic purity by HPLC

Purity of standard substances can be checked by HPLC as follows:

Inject appropriate volumes of PM, PL and PN standard solutions I (4.20.1, 4.20.2, 4.20.3) into the HPLC system and analyse as described in 6.4.

Calculate purity of the standard substances according to equation (2):

$$R_i = \frac{x_i \times 100}{x_i + B} \qquad (2)$$

where

R_i is the purity of standard substance *i* in %;

x_i is the peak area of standard substance *i;*

B is the sum of the peak areas of contaminating substances (without solvent peak).

The chromatographic purity of standard substances should be ≥ 98 %, otherwise take new standard substances or prepare new standard solutions.

4.21 Mixed calibration solution e. g. ρ(PM, PL, PN) = 0,1 µg/ml to 10 µg/ml

Pipette suitable volumes of PM, PL and PN stock solutions (4.19.1 to 4.19.3) or standard solutions (4.20.1 to 4.20.6) into a 20 ml volumetric flask, dilute with 0,1 mol/l HCl (4.8) to 6,5 ml, if necessary. Adjust to pH = 4,8 with 2,5 mol/l sodium acetate solution (4.5), and then adjust to pH = 3,0 with sulfuric acid (4.10), dilute with water to the mark and mix (calibration solutions). At least three calibration points are recommended. If necessary, the mixed calibration solutions may be diluted with mobile phase prior to HPLC injection.

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 devices

Laboratory autoclave and oven or water bath, with stirring facilities, able to be controlled at 37 °C

5.4 High performance liquid chromatographic system

Consisting of a pump, sample injecting device, fluorescence detector with excitation and emission wavelengths set at 290 nm and 390 nm, respectively and an evaluation system such as an integrator, and optionally, a post column derivatisation device

5.5 HPLC-Column, e. g. reversed phase column, such as:

Luna™ RP C_{18}, 5 µm[1)], particle size of 5 µm, diameter 4,0 mm, length 250 mm[2)]. Other suitable examples are listed in Annex B

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

Cut and 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. After homogenising, analyse the sample immediately.

6.2 Preparation of the sample test solution

6.2.1 Extraction

6.2.1.1 General

For samples with a high fat content (> 25 %) it can be useful to remove fat e.g. by repeated treatment with light petroleum before the acid hydrolysis.

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2) Other particle sizes or column dimensions than specified in this document 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.