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**FOODSTUFFS - DETERMINATION OF
VITAMIN B6 BY HPLC**

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EUROPEAN PRESTANDARD
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EUROPÄISCHE VORNORM

ENV 14164

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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

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

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ENV 14164:2002 (E)

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 B₆ in foodstuffs by HPLC.

Vitamin B₆ 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.

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).

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.

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, $w(\text{CH}_3\text{COONa} \cdot 3 \text{H}_2\text{O}) \geq 99,0 \%$

4.2.3 Glacial acetic acid, $w(\text{CH}_3\text{COOH}) \geq 99,8 \%$

4.2.4 Glyoxylic acid, $w(\text{C}_2\text{H}_2\text{O}_3 \cdot \text{H}_2\text{O}) \geq 97,0 \%$

4.2.5 Ferrous sulfate II, heptahydrate, $w(\text{FeSO}_4 \cdot 7\text{H}_2\text{O}) \geq 99,5 \%$

4.2.6 Sodium hydroxide, $w(\text{NaOH}) \geq 98,0 \%$

4.2.7 Sodium borohydride, $w(\text{NaBH}_4) \geq 97,0 \%$

4.2.8 Potassium dihydrogen phosphate, $w(\text{KH}_2\text{PO}_4) \geq 99,0 \%$

4.2.9 Orthophosphoric acid, $w(\text{H}_3\text{PO}_4) \geq 84,0 \%$

4.2.10 Sodium octanesulfonate, $w(\text{C}_8\text{H}_{17}\text{NaO}_3\text{S}) \geq 98,0 \%$, or sodium heptanesulfonate, $w(\text{C}_7\text{H}_{15}\text{NaO}_3\text{S}) \geq 98,0 \%$

4.2.11 Acetonitrile (HPLC grade), $w(\text{C}_2\text{H}_3\text{N}) \geq 99,8 \%$

4.2.12 Sodium acetate solution, $c(\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}) = 2,5 \text{ mol/l}$

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

4.2.13 Sodium acetate solution, $c(\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}) = 0,05 \text{ mol/l}$ (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, $c(\text{FeSO}_4 \cdot 7\text{H}_2\text{O}) = 0,0132 \text{ mol/l}$

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, $c(\text{NaOH}) = 0,2 \text{ mol/l}$

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

4.2.16 Sodium borohydride solution, $c(\text{NaBH}_4) = 0,1 \text{ mol/l}$

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, $c(\text{C}_2\text{H}_2\text{O}_3 \cdot \text{H}_2\text{O}) = 1 \text{ mol/l}$ (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.

ENV 14164:2002 (E)**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 B₆ standard substance

Pyridoxine hydrochloride, w ($\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$) $\geq 99 \%$

4.4 Stock solution**4.4.1 Vitamin B₆ stock solution, ρ ($\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$) $\approx 0,5$ mg/ml**

Dissolve an accurately weighed amount of the vitamin B₆ 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

Dilute 0,5 ml of vitamin B₆ 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):

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

where

A is the absorbance value of the solution at 290 nm;

M_w is the molecular weight of vitamin B₆ 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 $\text{mmol}^{-1} \text{cm}^{-1}$;

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

4.5 Standard solutions**4.5.1 Vitamin B₆ intermediate standard solution, ρ ($\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$) ≈ 10 $\mu\text{g/ml}$**

Pipette 1 ml of the vitamin B₆ 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.

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