SVENSK STANDARD
SS-EN 14166:2009

Fastställd/Approved: 2009-06-05
Publicerad/Published: 2009-08-18
Utgåva/Edition: 1
Språk/Language: engelska/English
ICS: 07.100.30

Foodstuffs – Determination of vitamin B6 by microbiological assay

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Denna standard ersätter SS-ENV 14166, utgåva 1.

The European Standard EN 14166:2009 has the status of a Swedish Standard. This document contains the official English version of EN 14166:2009.

This standard supersedes the Swedish Standard SS-ENV 14166, edition 1.

Upplysningar om sakinnehållet i standarden lämnas av SIS, Swedish Standards Institute, telefon 08-555 520 00.
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EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 14166

May 2009

ICS 07.100.30

Supersedes ENV 14166:2001

English Version

Foodstuffs - Determination of vitamin B6 by microbiological assay

Produits alimentaires - Détermination de la vitamine B6 par essai microbiologique

Lebensmittel - Mikrobiologische Bestimmung von Vitamin B6

This European Standard was approved by CEN on 23 April 2009.

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EUROPÄISCHES KOMITEE FÜR NORMUNG

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Ref. No. EN 14166:2009: E

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Foreword

This document (EN 14166:2009) 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of an identical text or by endorsement, at the latest by November 2009, and conflicting national standards shall be withdrawn at the latest by November 2009.

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1 Scope

This European Standard specifies a method for the determination of total vitamin B_6 in foodstuffs by microbiological assay (MBA). Vitamin B_6 is determined as the mass fraction of pyridoxine, pyridoxal and pyridoxamine, including their phosphorylated or glycosylated derivatives. It is usually expressed as milligram vitamin B_6 per 100 g of foodstuff. The method is applicable to samples that can be rendered homogeneous and do not contain high concentrations of antibiotics or other interfering substances.

This method has been validated in an inter-laboratory test on fortified and non-fortified samples such as wholemeal flour, milk powder, mixed vegetables and pigs liver at levels from 0,5 mg/100 g to 1,9 mg/100 g. For further information on the validation data, see Annex B.

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 3696:1995, *Water for analytical laboratory use — Specification and test methods (ISO 3696:1987)*

3 Principle

Pyridoxine, pyridoxal and/or pyridoxamine are extracted from foodstuffs by acid hydrolysis. The hydrolysis step liberates the vitamin B_6 vitamers from proteins and carbohydrates in the sample and hydrolyses the phosphates to the free vitamers. The total vitamin B_6 content in the sample extract is then determined by comparing the growth response of the assay test organism against growth obtained from a pyridoxine hydrochloride standard, see [1].

4 Reagents

4.1 General

During analysis, unless otherwise stated, use only reagents of recognised analytical grade and water of at least grade 1 according to EN ISO 3696:1995. The water used for reagent preparation shall be glass distilled. Once distilled, water shall be used within five days or discarded.

4.2 Chemicals and solutions

4.2.1 Sulfuric acid solution, substance concentration $c(H_2SO_4)$ = 0,22 mol/l

4.2.2 Sodium hydroxide solution, c(NaOH) = 4 mol/l

4.2.3 Wort agar, (Difco[1)] or suitable alternative)

Dissolve the agar in glass distilled water according to the manufacturer's instructions. Heat to boil. Dispense 5 ml aliquots into glass bottles, cap and autoclave at 121 °C for 15 min. Cool at an angle for slopes to form. Store in a refrigerator for up to three months.

1) This information is given for the convenience of users of this European Standard 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.

4.2.4 Basal medium (Difco Pyridoxine Y Medium[1)] or suitable alternative)

The concentration of the assay medium should be chosen, depending upon the assay format used, to ensure that the manufacturer's recommended concentration is obtained in the final assay volume.

4.2.5 Liquid culture medium

Dilute basal medium (4.2.4) with an equal volume of water containing 2,0 ng/ml pyridoxine, pyridoxamine and pyridoxal. Add 10 ml portions to screw-topped tubes and autoclave at 121 °C for 5 min and cool rapidly. Store in refrigerator for up to one month.

4.2.6 Inoculum rinse

Dilute basal medium (4.2.4) with an equal volume of water. Add 10 ml portions to screw-topped tubes and autoclave at 121 °C for 5 min and cool rapidly. Store in refrigerator for up to one month.

4.2.7 Sodium chloride, mass fraction $w(NaCl) \geq 98,0$ %

4.2.8 Sterile saline solution

Dissolve 0,9 g of sodium chloride (4.2.7) in 100 ml of glass distilled water. Autoclave at 121 °C for 15 min.

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

4.2.10 Sodium hydroxide solution, $c(NaOH) = 0,1$ mol/l

4.3 Test organism, *Saccharomyces uvarum* ATCC 9080 (freeze-dried yeast)

4.3.1 Test organism maintenance (stock culture)

The test organism is maintained by weekly transfers onto agar maintenance medium (4.2.3) using the following procedure:

Prepare 50 ml portions of pyridoxine basal medium (4.2.4) and place in a 100 ml thick-walled glass bottle or suitable flask. Add 2 ml of pyridoxine calibration solution 20 (4.8.1), cap and autoclave at 121 °C for 5 min. Cool as rapidly as possible in cold water to below 30 °C. Aseptically, add 1 ml of autoclaved medium to the freeze dried culture (4.3) and add 0,5 ml of the resultant suspension to the remaining medium using a sterile pipette. Incubate at 30 °C for 16 h. After incubation, the organism should show thick growth. Transfer the medium to suitable sterile, centrifuge tubes and centrifuge at 2000 g for 5 min. Discard the supernatant and wash the cell residue with two 50 ml portions of sterile saline (4.2.8), centrifuging between washes. Re-suspend the cells in 50 ml of sterile saline solution.

Using a sterile loop, transfer cells from this suspension onto three agar slopes (4.2.3) in a cross pattern and incubate for 16 h to 20 h at 30 °C. After incubation, the cross should show visible growth and there should be no growth in the surrounding areas. Store the organism in a refrigerator. The organism should be transferred to fresh agar slopes on a weekly basis. It is essential to maintain aseptic conditions during preparation and transfer of solutions.

4.3.2 Working inoculum

On the day before required, transfer cells from the stock culture (4.3.1) into two tubes of the liquid culture medium (4.2.5) keeping the transfers as sterile as possible. Incubate for 16 h to 20 h at 30 °C. Under aseptic conditions, centrifuge culture at 2000 g for 2 min and decant supernatant. Cells can be washed with 2 x 10 ml inoculum rinse (4.2.6) discarding the supernatant each time. Re-suspend cells in a third 10 ml portion of inoculum rinse. This is used for the assay inoculum.

4.4 Standard substance

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

4.5 Stock solution

4.5.1 Pyridoxine stock solution, $\rho(C_8H_{11}NO_3) \approx 200$ µg/ml

Accurately weigh 121,5 mg to the nearest 0,1 mg pyridoxine hydrochloride (4.4.1) in a small beaker. Dissolve in glass distilled water, then transfer quantitatively to a 500 ml volumetric flask. Dilute to the mark with glass distilled water and mix. This solution is stable for two weeks if kept refrigerated.

4.6 Concentration test

Dilute 2 ml of the stock solution (4.5.1) to 20 ml with 0,1 mol/l HCl. Measure the absorbance value at 290 nm against 0,1 mol/l HCl solution (pH ≈ 1).

Calculate the mass concentration, ρ, in µg/ml of the stock solution according to equation (1):

$$\rho = \frac{A \times M_w \times V}{\varepsilon} \qquad (1)$$

where:

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

ε is the molar extinction coefficient in a hydrochloric acid solution of c(HCl) = 0,1 mol/l at λ_{max} = 290 nm (here: 8 400 $mmol^{-1}cm^{-1}$, see [2]);

M_w is the molar mass of the standard substance, in gram per mol;

V is the dilution factor, i.e. 10.

4.7 Intermediate pyridoxine calibration solution, $\rho(C_8H_{11}NO_3) \approx 400$ ng/ml

Dilute 2 ml of pyridoxine stock solution (4.5.1) to 1000 ml with glass distilled water. Prepare on day of use.

4.8 Calibration solutions

4.8.1 Pyridoxine calibration solution 20, $\rho(C_8H_{11}NO_3) = 20$ ng/ml

Dilute 5 ml intermediate calibration solution (4.7) to 100 ml with glass distilled water. Prepare on day of use.

4.8.2 Pyridoxine calibration solution 10, $\rho(C_8H_{11}NO_3) = 10$ ng/ml

Dilute 25 ml of pyridoxine calibration solution 20 (4.8.1) to 50 ml with glass distilled water. Prepare on day of use.

4.8.3 Pyridoxine calibration solution 5, $\rho(C_8H_{11}NO_3) = 5$ ng/ml

Dilute 25 ml of pyridoxine calibration solution 20 (4.8.1) to 100 ml with glass distilled water. Prepare on day of use.

NOTE Alternative concentrations may be prepared to suit the assay format to be used, see 6.3.1 and 6.3.2.

5 Apparatus

5.1 General

Usual laboratory equipment and glassware. All glassware shall be washed with detergents that will not stimulate or depress the growth of the assay test organism. Glassware shall be thoroughly washed and rinsed with glass distilled water. If glass test tubes are used for the assay format, they should be thoroughly washed and then heated at a minimum temperature of 160 °C overnight before use. The following items are also required.

5.2 Autoclave, or similar heating device

5.3 UV spectrometer

5.4 Incubator or water-bath, capable of maintaining a constant and defined temperature and with a shaking facility.

5.5 Sterile pipettes and/or syringes

5.6 Autoclavable bottles or flasks

5.7 Sterile bottles or tubes

6 Procedure

6.1 Preparation of the test sample

The test sample shall be homogeneous. Coarse material shall be rendered homogeneous using an appropriate mill and/or blender. Pre-cooling of the sample may be necessary to prevent exposure to high temperatures.

6.2 Preparation of the test sample solution

Weigh an appropriate amount of sample (between 0,5 g and 10 g corresponds to about 2 µg of vitamin B_6) into a screw top glass bottle or conical flask, to the nearest 1 mg. Add 150 ml of sulfuric acid solution (4.2.1) and swirl the contents to mix. Cap the bottle (or flask) and autoclave at 121 °C for 5 h. Cool to room temperature in cold water, add 15 ml of sodium hydroxide solution (4.2.2) and cool again. Adjust the pH of the sample solution to 4,5 ± 0,1 using sodium hydroxide solution (4.2.10) and a pH meter. Transfer the solution to a 200 ml volumetric flask and dilute to the mark with glass distilled water. Mix thoroughly by inversion and filter through a fine porosity filter paper. Dilute with glass distilled water to an analyte concentration suitable for the assay calibration range used. A reagent blank should be run with every batch of samples.

6.3 Determination of vitamin B_6 by microbiological assay

6.3.1 Assay configuration - Standards

The growth response of the different vitamin B_6 vitamers to the assay organism varies (see Annex A). Pyridoxal shows a slightly lower dose response than pyridoxine and pyridoxamine shows an even lower dose response (e.g. pyridoxine for plant based foods, pyridoxal for dairy based foods, pyridoxal or pyridoxamine for meats). It is common practice to use pyridoxine as calibrant but this may cause an underestimate where significant levels of pyridoxamine are present.