# SLOVENSKI STANDARD
# SIST ENV 14166:2002

**01-junij-2002**

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**Živila - Določevanje vitamina B6 z mikrobiološko analizo**

Foodstuffs - Determination of vitamin B6 by microbiological assay

Lebensmittel - Mikrobiologische Bestimmung von Vitamin B6

Produits alimentaires - Dosage de la vitamine B6 par essai microbiologique



**Ta slovenski standard je istoveten z:** **ENV 14166:2001**

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# ENV 14166

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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 Prestandard (ENV) was approved by CEN on 29 September 2001 as a prospective standard for provisional application.

The period of validity of this ENV is limited initially to three years. After two years the members of CEN will be requested to submit their comments, particularly on the question whether the ENV can be converted into a European Standard.

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

**Management Centre: rue de Stassart, 36 B-1050 Brussels**



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# 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.

Annexes A and B are informative.

This standard includes a Bibliography.

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, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

## 1 Scope

This European Prestandard 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.

## 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 3696:1995, *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 $B_6$ 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 appropriate standards, 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 as defined in 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 $c$ ($H_2S0_4$) = 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.

**4.2.4 Basal medium** (Difco, Pyridoxine Y assay 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) ≥ 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, *Sacchromyces 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 solution (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 fridge. 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.

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

#### 4.3.2 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. Wash cells 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 substances

#### 4.4.1 Pyridoxine hydrochloride, $w$ ($C_8H_{11}NO_3.HCl$) ≥ 98 %

#### 4.4.2 Pyridoxal hydrochloride, $w$ ($C_8H_9NO_3.HCl$) ≥ 98 %

#### 4.4.3 Pyridoxamine dihydrochloride, $w$ ($C_8H_{12}N_2O_2.2HCl$) ≥ 98 %

### 4.5 Stock solutions

#### 4.5.1 Pyridoxine stock solution, $\rho$ ($C_8H_{11}NO_3$) ≈ 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.

#### 4.5.2 Pyridoxal stock solution, $\rho$ ($C_8H_9NO_3$) ≈ 200 µg/ml



Prepare as for Pyridoxine using 121,8 mg to the nearest 0,1 mg pyridoxal hydrochloride (4.4.2). This solution is stable for two weeks if kept refrigerated.



#### 4.5.3 Pyridoxamine stock solution, $\rho$ ($C_8H_{12}N_2O_2$) ≈ 200 µg/ml



Prepare as for Pyridoxine using 143,4 mg to the nearest 0,1 mg pyridoxamine dihydrochloride (4.4.3). This solution is stable for two weeks if kept refrigerated.

### 4.6 Concentration test

Depending on the standard substance used, dilute 2 ml of the appropriate stock solution (4.5.1 to 4.5.3) to 20 ml with 0,1 mol/l HCl. Measure the absorbance at the wavelengths shown in Table 1, against 0,1 mol/l HCl solution (pH ~ 1).

**Table 1 — Concentration test parameters [2]**

| Standard | Solvent | $\lambda_{max}$ | Molar Extinction Coeff. ε |
|---|---|---|---|
| Pyridoxine·HCl | 0, 1 mol/l HCl | 290 | 8400 |
| Pyridoxal·HCl | 0,1mol/l HCl | 288 | 9000 |
| Pyridoxamine·2 HCl | 0,1 mol/l HCl | 292 | 8200 |

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

$$\rho = \frac{A \cdot M_W \cdot V}{\varepsilon} \qquad (1)$$

where:

$A$ is the absorbance value of the solution at the relevant wavelength;

ε is the appropriate molar extinction coefficient from Table 1;

$M_w$ is the molecular weight of the standard substance, in gram per mol;

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

## 4.7 Intermediate calibration solutions, $\rho \approx 400$ ng/ml.

### 4.7.1 Pyridoxine intermediate calibration solution, $\rho$ ($C_8H_{11}NO_3$) ≈ 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.

### 4.7.2 Pyridoxal intermediate calibration solution, $\rho$ ($C_8H_9NO_3$) ≈ 400 ng/ml.

Dilute 2 ml of Pyridoxal stock solution (4.5.2) to 1000 ml with glass distilled water. Prepare on day of use.

### 4.7.3 Pyridoxamine intermediate calibration solution, $\rho$ ($C_8H_{12}N_2O_2$) ≈ 400 ng/ml.

Dilute 2 ml of Pyridoxamine stock solution (4.5.3) 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 (4.7.1) 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 20 ng/ml calibration solution (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 20 ng/ml calibration solution (4.8.1) to 100 ml with glass distilled water. Prepare on day of use.

### 4.8.4 Pyridoxal calibration solution 20, $\rho$ ($C_8H_9NO_3$) = 20 ng/ml

Dilute 5 ml intermediate calibration solution (4.7.2) to 100 ml with glass distilled water. Prepare on day of use.

### 4.8.5 Pyridoxal calibration solution 10, $\rho$ ($C_8H_9NO_3$) = 10 ng/ml

Dilute 25 ml of 20 ng/ml calibration solution (4.8.4) to 50 ml with glass distilled water. Prepare on day of use.

### 4.8.6 Pyridoxal calibration solution 5, $\rho$ ($C_8H_9NO_3$) = 5 ng/ml

Dilute 25 ml of 20 ng/ml calibration solution (4.8.4) to 100 ml with glass distilled water. Prepare on day of use.

### 4.8.7 Pyridoxamine calibration solution 40, $\rho$ ($C_8H_{12}N_2O_2$) = 40 ng/ml

Dilute 10 ml intermediate calibration solution (4.7.3) to 100 ml with glass distilled water. Prepare on day of use.

### 4.8.8 Pyridoxamine calibration solution 20, $\rho$ ($C_8H_{12}N_2O_2$) = 20 ng/ml

Dilute 25 ml of 40 ng/ml calibration solution (4.8.7) to 50 ml with glass distilled water. Prepare on day of use.

### 4.8.9 Pyridoxamine calibration solution 10, $\rho$ ($C_8H_{12}N_2O_2$) = 10 ng/ml

Dilute 25 ml of 40 ng/ml calibration solution (4.8.7) 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 sample test solution

Weigh an appropriate amount of sample (between 0,5 g and 10 g {≅ 2 µg $B_6$}) 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 $B_6$ 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. Separate calibration lines should be obtained for pyridoxine, pyridoxal and pyridoxamine. The calibration line for the vitamer which is predominant in the sample should be used (e.g. Pyridoxine for plant based foods, pyridoxal for dairy based foods, pyridoxal or pyridoxamine for meats). For unknown samples, the calibration line, which most closely resembles the dose response curve for the sample, should be used. It is common practice to use pyridoxine as the primary calibrant but this may cause an underestimate where significant levels of pyridoxamine are present.

Microbiological assays may be performed in test tubes or in microtitre-plate format. The volumes of standards, sample extracts, and assay media used will vary between laboratories depending upon assay format. Two assay formats, which use ‘low’ and ‘high’ volumes of test sample extract respectively, are provided in Tables 2 and 3 below.

Depending on the assay format (see Tables 2 and 3), pipette the appropriate amount of the calibration solutions (4.8.1 to 4.8.9) into assay tubes. Pipette all assay standards in duplicate (two tubes for each level of standard).

tubes 1 and 2 are the uninoculated blank and tubes 3 and 4 are the inoculated blank. Add the appropriate amount of assay medium (4.2.4) and if appropriate, water to each tube. Prepare similar tubes for pyridoxine, pyridoxal and pyridoxamine as required.

**Table 2 — Example format 1 – Standards**

| Tube number | 0 – 2 | 3 – 4 | 5 – 6 | 7 – 8 | 9 – 10 | 11- 12 | 13 – 14 | 15 – 16 | 17 – 18 | 19 – 20 | 21 – 22 | 23 – 24 | 25 – 26 | 27 - 28 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | |
| Water, ml | 0,4 | 0,4 | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| PN Std (20 ng/ml), ml | | | 0,1 | 0,2 | 0,3 | 0,4 | | | | | | | | |
| *ng PN per tube* | | | *2* | *4* | *6* | *8* | | | | | | | | |
| | | | | | | | | | | | | | | |
| PN Std (10 ng/ml), ml | | | | | | | 0,1 | 0,2 | 0,3 | 0,4 | | | | |
| *ng PN per tube* | | | | | | | *1* | *2* | *3* | *4* | | | | |
| | | | | | | | | | | | | | | |
| PN Std (5 ng/ml), ml | | | | | | | | | | | 0,1 | 0,2 | 0,3 | 0,4 |
| *ng PN per tube* | | | | | | | | | | | *0,5* | *1* | *1,5* | *2* |
| | | | | | | | | | | | | | | |
| Assay Media, ml | 9,6 | 9,6 | 9,9 | 9,8 | 9,7 | 9,6 | 9,9 | 9,8 | 9,7 | 9,6 | 9,9 | 9,8 | 9,7 | 9,6 |
| | | | | | | | | | | | | | | |
| Total Volume, ml | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |



**Table 3 — Example format 2 - Standards**

| Tube number | 0 – 2 | 3 – 4 | 5 – 6 | 7 – 8 | 9 – 10 | 11- 12 | 13 – 14 | 15 – 16 | 17 – 18 | 19 – 20 | 21 – 22 | 23 – 24 | 25 – 26 | 27 - 28 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | |
| Water, ml | 4 | 4 | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| PN Std (2 ng/ml), ml | | | 1 | 2 | 3 | 4 | | | | | | | | |
| *ng PN per tube* | | | *2* | *4* | *6* | *8* | | | | | | | | |
| | | | | | | | | | | | | | | |
| PN Std (1 ng/ml), ml | | | | | | | 1 | 2 | 3 | 4 | | | | |
| *ng PN per tube* | | | | | | | *1* | *2* | *3* | *4* | | | | |
| | | | | | | | | | | | | | | |
| PN Std (0,5 ng/ml), ml | | | | | | | | | | | 1 | 2 | 3 | 4 |
| *ng PN per tube* | | | | | | | | | | | *0,5* | *1* | *1,5* | *2* |
| | | | | | | | | | | | | | | |
| Water, ml | 1 | 1 | 4 | 3 | 2 | 1 | 4 | 3 | 2 | 1 | 4 | 3 | 2 | 1 |
| | | | | | | | | | | | | | | |
| Double Strength Assay Media, ml | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| | | | | | | | | | | | | | | |
| Total Volume, ml | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 10 |



### 6.3.2 Assay configuration - Test sample extracts

Depending on the assay format, pipette the appropriate amount of the test sample extract (6.2) in duplicate, into test tubes as described in Tables 4 and 5. These shall match the format used for standards (6.3.1). Standards and samples shall be assayed at the same time and under the same conditions. Sample extract dilution is dependent