BRITISH STANDARD

BS EN 14166:2009

Foodstuffs — Determination of vitamin B6 by microbiological assay

ICS 07.100.30

BSI British Standards

National foreword

This British Standard is the UK implementation of EN 14166:2009. It supersedes DD ENV 14166:2001 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee AW/-/3, Food analysis - Horizontal methods.

A list of organizations represented on this committee can be obtained on request to its secretary.

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Foodstuffs - Determination of vitamin B6 by microbiological assay

Produits alimentaires - Détermination de la vitamine B6 par essai microbiologique

Lebensmittel - Mikrobiologische Bestimmung von Vitamin B6

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of an identical text or by endorsement, at the latest by November 2009, and conflicting national standards shall be withdrawn at the latest by November 2009.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

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

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.

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 1 and 2 below.

Depending on the assay format (see Tables 1 and 2), pipette the appropriate amount of the calibration solutions (4.8.1 to 4.8.3) 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 as required.

Table 1 — 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 2 — 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 3 and 4. 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 upon the format used and shall be determined so that the sample dose response curve is similar to the standard range.

Table 3 — Example format 1 – Test sample solutions

Tube number	29 - 30	31 - 32	33 - 34	35 – 36
Sample test extract	0,10 ml	0,20 ml	0,30 ml	0,40 ml
Assay media	9,9 ml	9,8 ml	9,7 ml	9,6 ml

Table 4 — Example format 2 - Test sample solutions

Tube number	29 - 30	31 - 32	33 - 34	35 – 36
Sample test extract	1,0 ml	2,0 ml	3,0 ml	4,0 ml
Water	4,0 ml	3,0 ml	2,0 ml	1,0 ml
Assay media double strength	5,0 ml	5,0 ml	5,0 ml	5,0 ml

This pattern is repeated in subsequent tubes for further samples.

6.3.3 Sterilization of standards and test sample extracts

Cover the tops of all test tubes (from 6.3.1 and 6.3.2) with caps to prevent bacterial contamination, and autoclave all tubes at 121 °C for 5 min. Cool tubes as rapidly as possible in cold running water to ambient temperature.

6.3.4 Inoculation of the assay

Aseptically inoculate each test tube, with the exception of the 'blanks' in tube 1 and 2 which act as uninoculated blanks, by addition of 100 µl of inoculum (4.3.2) using a sterile syringe pipette or similar. Mix the contents of each tube after inoculation. The contents of the tubes shall be below 30 ^{o}C prior to inoculation.

6.3.5 Assay incubation

Incubate the assay test tubes at 30 °C ± 0,5 °C for 18 h to 24 h in a waterbath. A waterbath with a shaking facility is recommended.

Cool the assay tubes in running cold water after the incubation period and prior to reading the optical density.

6.3.6 Measurement of optical density

Mix the contents of each tube (e.g. by vortex mixer) and measure the optical density in the visible region of the spectrum (e.g. 550 nm to 600 nm). This may be achieved by direct measurement by insertion of assay tubes into an appropriate spectrometer, by transfer of an aliquot of assay medium into a cuvette and measurement, or by use of a robotised process. Microtitre plates are read via an automated microtitre-plate reader. The time interval between tube mixing and optical density measurement should be standardised for all tubes.

7 Calculation

7.1 Calibration

Duplicate data will be obtained for all standards. The uninoculated blank should be clear. The inoculated blanks should also be clear or show only a very small amount of turbidity. If the inoculated blanks are satisfactory, read the absorbance of the remaining tubes. Plot the absorbance of each tube against the

concentration of standard in the corresponding assay tube and construct a calibration line. Annex A shows typical calibration lines obtained.

7.2 Test samples

Duplicate data will be obtained for all samples at each dilution level. Interpolate the amount of analyte in the test sample aliquot from the calibration line. Calculations can be performed manually or using an appropriate computer programme.

7.3 Calculation of the mass fraction of vitamin B_6 in the test sample

Calculate the mass fraction, ρ, of vitamin B_6 in mg/100 g in the test sample according to Equation (2)

$$\rho = \frac{m_{B_6} \times V_s}{V_{ts} \times m_s \times 10^6} \times 100 \qquad (2)$$

where:

m_{B6} is the amount of vitamin B_6 in the assay tube (mean of duplicate readings) at the sample test solution level, in nanogram;

V_s is the final total volume of the sample test solution (6.2), in millilitre;

V_{ts} is the volume of sample test solution pipetted into the assay tube, in millilitre;

m_s is the mass of the test sample taken for analysis, in gram;

100 is the multiplier to obtain the result per 100 g;

10^6 is the factor to obtain the result in milligram (conversion from nanogram).

Calculate the result of vitamin B_6 in mg/100 g in the test sample from the mean value of all dilution levels. The result is expressed as the microbiological activity referred to the pyridoxine hydrochloride (4.4), which was used for calibration.

Calculations in 7.3 may be performed simultaneously with operations outlined in 7.1 and 7.2, using a suitable computer programme.

8 Criteria for acceptance of data

Criteria for acceptance of data may be assay-format dependent and shall be established in each laboratory. As a minimum, the following criteria should be considered:

a) the maximum turbidity of the inoculated blank solutions;

b) the minimum turbidity of the most concentrated calibration solution;

c) variation between replicate absorbance values obtained at each calibration level;

d) variation of calculated amounts of vitamin B_6 as pyridoxin between all dilutions of test sample solution;

e) analysis of relevant reference control samples as available.

9 Precision

9.1 Statistical summary

Details of the inter-laboratory test of precision of the method are summarised in Annex B. The values derived from this test may not be applicable to analyte concentration ranges and test sample matrices other than those given in Annex B.

9.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit *r*, in not more than 5 % of the cases.

The values for pyridoxine in mg/100 g dry matter are:

Wholemeal flour	$\bar{x}$ = 0,45 mg/100 g	*r* = 0,08 mg/100 g
Milk Powder	$\bar{x}$ = 0,65 mg/100 g	*r* = 0,11 mg/100 g
Mixed Vegetables	$\bar{x}$ = 0,46 mg/100 g	*r* = 0,07 mg/100 g
Pigs Liver	$\bar{x}$ = 1,87 mg/100 g	*r* = 0,50 mg/100 g

9.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit *R*, in not more than 5 % of the cases.

The values for pyridoxine in mg/100 g dry matter are:

Wholemeal flour	$\bar{x}$ = 0,45 mg/100 g	*R* = 0,36 mg
Milk Powder	$\bar{x}$ = 0,65 mg/100 g	*R* = 0,29 mg
Mixed Vegetables	$\bar{x}$ = 0,46 mg/100 g	*R* = 0,29 mg
Pigs Liver	$\bar{x}$ = 1,87 mg/100 g	*R* = 1,37 mg

10 Test Report

The test report shall contain at least the following data:

a) all information necessary for the complete identification of the sample;

b) a reference to this European Standard or to the method used;

c) date of sample receipt;

d) date of test;

e) the results and the units in which the results have been expressed;

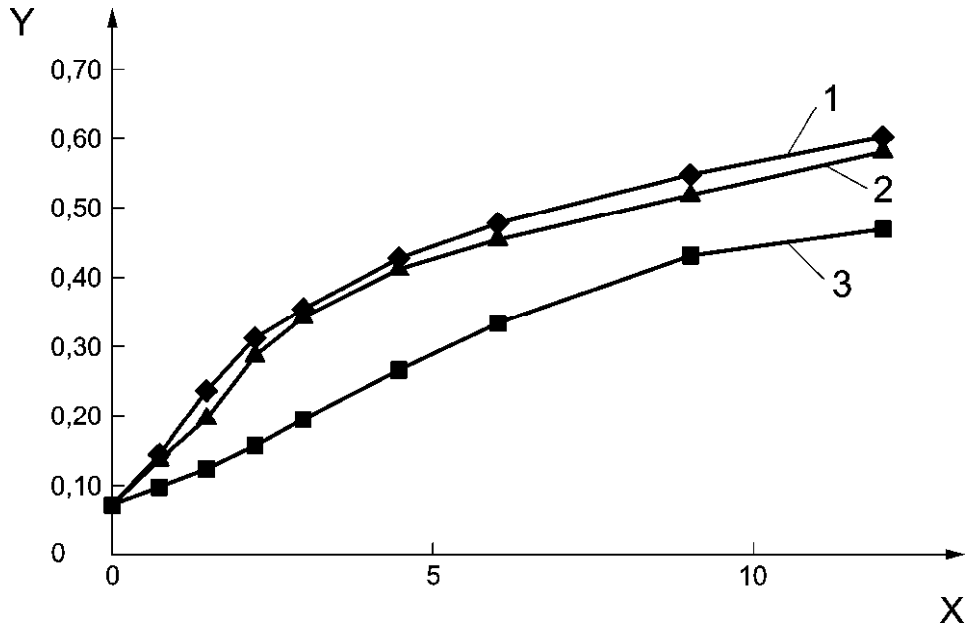
f) any particular points observed in the course of the test;

g) any operations not specified in the method or regarded as optional which might have affected the results;

h) date of test report issue, name and signature of the responsible analyst.

Annex A
(informative)

Vitamin B_6 calibration lines obtained by MBA using the test organism *Saccharomyces uvarum*



Key:
x-axis: ng/tube
y-axis: absorbance
1: pyridoxine
2: pyridoxal
3: pyridoxamine

Figure A.1 — Dose response of *Saccharomyces Uvarum* to B_6 vitamers

Annex B
(informative)

Precision data

The following data were obtained in an inter-laboratory trial held in 1996 between participating European laboratories, see [3].

Table B.1 — Precision data

Sample	Wholemeal flour	Milk powder	Mixed vegetables	Pigs liver
Year of study	1996	1996	1996	1996
Number of laboratories	7	7	7	7
Outliers	0	0	0	0
Mean value $\bar{x}$ (mg/100 g)	0,45	0,65	0,45	1,87
Repeatability standard deviation s_r (mg/100 g)	0,03	0,04	0,02	0,18
Repeatability relative standard deviation	6 %	6 %	5 %	9 %
Repeatability value r (95 %) (mg/100 g)	0,08	0,11	0,07	0,50
Reproducibility standard deviation s_R (mg/100 g)	0,13	0,10	0,10	0,49
Reproducibility relative standard deviation	28 %	15 %	23 %	26 %
Reproducibility value R (95 %) (mg/100 g)	0,36	0,28	0,29	1,37

Bibliography

[1] *Microbiological assay of vitamins of the B group*, Bell, J.G., Lab. Practice, 1974, 23, 235 - 242

[2] Metzler and Snell (1955): *Spectra and ionisation constants of the vitamin B_6 group and related 3-hydroxypyridine derivates.* Journal of the American Chemical Society 77: 2431 – 2437

[3] *The certification of the mass fractions of vitamins in four reference materials: wholemeal flour (CRM 121), milk powder (CRM 421), lyophilised mixed vegetables (CRM 485) and lyophilised pigs liver (CRM 487).* Finglas, P.M., Scott, K.J., Witthoft, C., van den Berg, H. & Froidmont-Görtz, I. (1999); EUR-report 18320, Office for Official Publications of the European Communities, Luxembourg.

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