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Foodstuffs — Determination of vitamin B6 by high performance chromatography

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National foreword

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The UK participation in its preparation was entrusted to Technical Committee AW/275, Food analysis - Horizontal methods.

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

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Lebensmittel - Bestimmung von Vitamin B6 mit Hochleistungs-Flüssigchromatographie

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Foreword

This document (EN 14164:2014) 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of an identical text or by endorsement, at the latest by December 2014 and conflicting national standards shall be withdrawn at the latest by December 2014.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. CEN [and/or CENELEC] shall not be held responsible for identifying any or all such patent rights.

This document supersedes EN 14164:2008.

The Annexes A, B, C and D are informative.

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1 Scope

This European Standard 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 determined as pyridoxine. The β-glycosylated forms are not taken into account. These can be determined with the method given in EN 14663 [1] by which the different vitamins of vitamin B_6 (pyridoxal, pyridoxamine and pyridoxine) are separated and individually quantified. A third European Standard, EN 14166 [2], determines the total vitamin B_6 by microbiological assay.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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)*

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 the presence of Fe^{2+} as a 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 [3], [4].

4 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of at least grade 1 according to EN ISO 3696, or double distilled water.

4.1 Acid phosphatase, (CAS 9001-77-8), from potatoes, enzyme activity is 33 nkat/mg[1)] with substrate p-nitrophenyl phosphate at pH = 4,8 and T = 37 °C, for example from Boehringer or Sigma[2)]. 33 nkat/mg corresponds to 2 U/mg.

4.1.1 Acid phosphatase solution

Prepare a solution of 20 mg/ml acid phosphatase in sodium acetate solution (4.14).

It is necessary to use acid phosphatase rather than Taka-diastase to obtain a complete hydrolysis of phosphorylated forms of vitamin B_6. Where 300 mg of Taka-diastase is needed to obtain good dephosphorylation, only 0,5 mg of acid phosphatase is needed, see [5].

1) Katal (symbol "kat") is a derived SI unit of enzyme activity. One katal is that catalytic activity which will raise the rate of reaction by one mol/s in a specified assay system.

2) This information is given for the convenience of users of this European standard and does not constitute an endorsement by CEN of the supplier. Equivalent products may be used if they can be shown to lead to the same results.

4.1.2 Activity check of acid phosphatase

The activity of acid phosphatase can be checked as proposed below.

Prepare a stock solution of approximately 0,1 mg/ml of pyridoxal phosphate (4.9) in water.

Mix 3,0 ml of the PLP stock solution and 10 ml of hydrochloric acid (4.21) in a 20 ml volumetric flask and fill up to the mark with water. Check the concentration of PLP by measuring the absorbance at 293 nm in a 1 cm cell using a UV-spectrometer (5.1) against a hydrochloric acid solution (4.20) as reference. The molar absorption coefficient (ε) of PLP in 0,1 mol/l HCl is 7 200.

Calculate the mass concentration ρ_{PLP} of the stock solution, in milligram per millilitre, according to Formula (1):

$$\rho_{PLP} = \frac{A_{293} \cdot M_{PLP}}{\varepsilon} \cdot F \quad (1)$$

where

- A_{293} is the absorption value of the solution at 293 nm;
- M_{PLP} is the molar mass of vitamin B_6 standard substance, in gram per mol (M_{PLP} = 247,14);
- F is the dilution factor (here F = 20/3);
- ε is the molar absorption coefficient of PLP in 0,1 mol/l of hydrochloric acid at 293 nm, in $l\ mol^{-1}\ cm^{-1}$, (here: ε = 7 200), see [6].

Take 1,0 ml of the PLP stock solution for extraction and proceed with 6.2.1, 6.2.2, 6.2.3 and 6.2.4.

Calculate the pyridoxine (PN) conversion rate from the dephosphorylated pyridoxal phosphate solution according to Formula (2):

$$\text{Conversion rate (\%)} = \frac{\rho_{PN\cdot HCl} \cdot A_S \cdot 2 \cdot 100 \cdot 0{,}822 \cdot 100 \cdot M_{PLP}}{A_{ST} \cdot 1\,000 \cdot \rho_{PLP} \cdot M_{PN}} \quad (2)$$

where

- $\rho_{PN\ HCL}$ is the mass concentration of pyridoxine hydrochloride in the standard test solution, in micrograms per millilitre;
- A_S is the peak area or peak height for pyridoxine obtained with the sample test solution, in units of area or height;
- 2 is the factor of dilution of the reaction with sodium borohydride if acetic acid is added, otherwise the dilution factor is 1,9;
- 100 is the total volume of the sample test solution, in millilitre;
- 0,822 is the factor to convert pyridoxine hydrochloride to pyridoxine;
- 100 is the conversion factor for %;
- M_{PLP} is the molar mass of pyridoxal phosphate (PLP), in gram per mol (M_{PLP} = 247,14);
- A_{ST} is the peak area or peak height for pyridoxine obtained with the standard test solution, in units of area or height;
- 1 000 is the factor to convert microgram to milligram;
- ρ_{PLP} is the mass concentration of pyridoxal phosphate (PLP) in the stock solution, in milligrams per millilitre;
- M_{PN} is the molar mass of pyridoxine (PN), in gram per mol (M_{PN} = 169,1).

4.2 Sodium acetate, trihydrate, mass fraction $w(CH_3COONa \cdot 3H_2O) \geq 99,0$ %.

4.3 Glacial acetic acid, $w(CH_3COOH) \geq 99,8$ %.

4.4 Glyoxylic acid, $w(C_2H_2O_3 \cdot H_2O) \geq 97,0$ %.

4.5 Ferrous sulfate II, heptahydrate, $w(FeSO_4 \cdot 7H_2O) \geq 99,5$ %.

4.6 Sodium hydroxide, $w(NaOH) \geq 99,0$ %.

4.7 Sodium borohydride, $w(NaBH_4) \geq 97,0$ %.

4.8 Potassium dihydrogen phosphate, $w(KH_2PO_4) \geq 99,0$ %.

4.9 Pyridoxal phosphate (PLP), $w \geq 99,0$ %.

4.10 Orthophosphoric acid, $w(H_3PO_4) \geq 84,0$ %.

4.11 Sodium octanesulfonate, $w(C_8H_{17}NaO_3S) \geq 98,0$ %, or sodium heptanesulfonate, $w(C_7H_{15}NaO_3S) \geq 98,0$ %.

4.12 Acetonitrile (HPLC grade), $w(C_2H_3N) \geq 99,8$ %.

4.13 Sodium acetate solution, substance concentration $c(CH_3COONa \cdot 3H_2O) = 2,5$ mol/l.

Dissolve 170,1 g of sodium acetate, trihydrate (4.2) in 500 ml of water.

4.14 Sodium acetate solution, $c(CH_3COONa \cdot 3H_2O) = 0,05$ mol/l (pH = 4,5).

Dissolve 6,8 g of sodium acetate, trihydrate (4.2) in 1 l of water. Adjust the pH to 4,5 with glacial acetic acid (4.3).

4.15 Ferrous sulfate solution, $c(FeSO_4 \cdot 7H_2O) = 0,0132$ mol/l.

Dissolve 36,6 mg of ferrous sulfate II, heptahydrate (4.5) in 10 ml of sodium acetate solution (4.14). Prepare fresh each day of use.

NOTE In a study described by Mann et al., see [7], a ferrous sulfate solution of 10 g/l was used. This concentration was based on the completion of the conversion of pyridoxamine to pyridoxal at pyridoxamine levels up to 8 times the minimum level of vitamin B_6 required by the infant formula Act in the US, see Mann et al. [8]. This concentration seems not to be necessary for the European situation.

4.16 Sodium hydroxide solution, $c(NaOH) = 0,2$ mol/l.

Dissolve 800 mg of sodium hydroxide (4.6) in 100 ml of water.

4.17 Sodium hydroxide solution, $c(NaOH) = 6,0$ mol/l.

Dissolve 24 g of sodium hydroxide (4.6) in 100 ml of water.

4.18 Sodium borohydride solution, $c(NaBH_4) = 0,1$ mol/l.

Dissolve 378 mg of sodium borohydride (4.7) in 100 ml of sodium hydroxide solution (4.16). Prepare fresh on day of use.

4.19 Glyoxylic acid solution, $c(C_2H_2O_3 \cdot H_2O) = 1$ mol/l (pH = 4,5).

Dissolve 4,7 g of glyoxylic acid monohydrate (4.4) in 30 ml of sodium acetate solution (4.13). Adjust the pH to 4,5 with the sodium hydroxide solution (4.17) and dilute to 50 ml with water in a volumetric flask. Prepare fresh on day of use.

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

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

4.22 HPLC mobile phase

In a beaker add 940 ml of water, 40 ml of acetonitrile (4.12), 160 mg of sodium octanesulfonate or sodium heptanesulfonate (4.11) and 6,8 g of potassium dihydrogen phosphate (4.8).

After dissolving sodium octanesulfonate or sodium heptanesulfonate and potassium dihydrogen phosphate by stirring, adjust the pH to 2,5 with orthophosphoric acid (4.10). Transfer the solution in a 1 l volumetric flask. Adjust to the mark with water. Filter through a 0,45 µm filter.

4.23 Pyridoxine hydrochloride (vitamin B_6 standard substance), $w(C_8H_{11}NO_3\ HCl) \geq 99$ %.

4.24 Pyridoxine hydrochloride stock solution, mass concentration $\rho \approx 0,5$ mg/ml.

Dissolve an accurately weighed amount of pyridoxine hydrochloride (4.23), e.g. 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.

For the concentration test, dilute 0,5 ml of pyridoxine hydrochloride stock solution (4.24) to 20 ml with 0,1 mol/l HCl (4.20) and measure the absorbance at 290 nm in a 1 cm cell using a UV-spectrometer (5.1) against 0,1 mol/l HCl solution as reference. Calculate the mass concentration ρ, in microgram per millilitre of the stock solution according to Formula (3):

$$\rho_{\text{PNHCl}} = \frac{A_{290} \cdot M_{\text{PNHCl}} \cdot 1\,000}{\varepsilon} \cdot F \tag{3}$$

where

A_{290}	is the absorption of the value of the solution at 290 nm;
M_{PNHCl}	is the molar mass of vitamin B_6 standard substance, in gram per mol (M_{PNHCl} = 205,64);
F	is the dilution factor (here F = 40);
ε	is the molar absorption coefficient of pyridoxine hydrochloride in 0,1 mol/l of hydrochloric acid at 291 nm, in $l\ mol^{-1}\ cm^{-1}$ (here ε = 8 600), see [9].

Further information on molar absorption coefficients in other solutions than 0,1 mol/l HCl (pH ≈ 1) is given in Annex D.

4.25 Standard solutions

4.25.1 Pyridoxine hydrochloride intermediate standard solution, $\rho(C_8H_{11}NO_3 \cdot HCl) \approx 10$ µg/ml.

Pipette 1 ml of the vitamin B_6 stock solution (4.24) into a 50 ml volumetric flask and dilute to the mark with water. Prepare this solution each day of analysis.

4.25.2 Pyridoxine hydrochloride standard test solution for HPLC, $\rho(C_8H_{11}NO_3 \cdot HCl) \approx 0,1$ µg/ml to 1 µg/ml.

Prepare a series of appropriate test standard solutions of concentrations ranging from e.g. 0,1 µg/ml to 1 µg/ml of pyridoxine hydrochloride by using the pyridoxine intermediate standard solution (4.25.1). Prepare these solutions fresh on day of use.

Perform a system suitability test by injecting a mixed standard test solution for HPLC of pyridoxine (PN) and pyridoxal (PL). PL and PN should have about the concentration in the standard test solution for HPLC. PL elutes before PN. The PN/PL pair shall have baseline separation. Add pyridoxamine (PM) as spike recovery in one sample run to ensure complete deamination and oxidation.

5 Apparatus

Usual laboratory apparatus, glassware, and, the following:

5.1 UV Spectrometer, capable of measurement of absorbance at defined wavelengths.

5.2 Heating device, oven or water bath, with shaking facilities.

5.3 High performance liquid chromatographic system, consisting of a pump, sample injecting device, fluorescence detector with excitation and emission wavelengths set at 290 nm and 395 nm, respectively and an evaluation system such as an integrator.

5.4 HPLC column, e.g. reversed phase column, such as LiChrospher® 60 RP C8 Select B [3)], particle size of 5 µm, diameter 4,0 mm, length 250 mm.

Other particle sizes or column dimensions than specified in this European Standard may be used. Separation parameters shall be adapted to such materials to guarantee equivalent results. The performance criterion for suitable analytical columns is the baseline resolution of the analytes concerned.

5.5 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

Homogenize the test sample. Grind coarse material with an appropriate mill and mix again. Measures such as pre-cooling shall be taken to avoid exposing to high temperature for long periods of time.

6.2 Preparation of the sample test solution

6.2.1 Extraction

Weigh an appropriate amount of the sample to the nearest milligram, e.g. 2,5 g (if the vitamin B_6 content exceeds 2 µg/g) or 5 g (if the vitamin content is less than 2 µg/g) in a conical flask. Add 50 ml of hydrochloric acid (4.20). Heat for 30 min in a boiling water bath.

NOTE 1 For samples, with a high water content or low contents of vitamin B_6, it can be useful to increase the sample weight, e.g. 20 g, to add an adapted volume of water, e.g. 25 ml, and to add directly 5 ml of hydrochloric acid *c* (HCl) = 1 mol/l.

[3)] LiChrospher® 60 RP C8 Select B is an example of a suitable product available commercially. This information is given for the convenience of users and does not constitute an endorsement by CEN of the product named. Equivalent products can be used if they lead to equivalent results.

NOTE 2 For samples with a high fat content it can be useful to remove fat with e.g. light petroleum before the acid hydrolysis.

NOTE 3 The main advantage of a preliminary acid hydrolysis is to improve the filtration step for samples with starch. The existing data in Annex B have been mainly obtained without any acid hydrolysis. A modification of the procedure (without acid hydrolysis) is described in Annex C.

6.2.2 Enzyme treatment and transformation steps

After cooling to room temperature, adjust the extract to pH 4,5 with sodium acetate solution (4.13) and add 2,5 ml of 1 mol/l glyoxylic acid solution (4.19), 400 µl of ferrous sulfate solution (4.15) and 1 ml of acid phosphatase solution (4.1.1). Incubate the solution overnight at 37 °C with continuous agitating.

After cooling to room temperature, dilute to 100 ml with water in a volumetric flask. Shake and filter. Mix 5,0 ml of filtrate and 4,5 ml of 0,1 mol/l sodium borohydride solution (4.18). Shake at least for 3 min. In order to ensure the complete destruction of the excess of sodium borohydride, it is possible to add 0,5 ml of glacial acetic acid (4.3). Take care for the dilution factor. Shake for 1 min. Filter through 0,45 µm filter. Use this filtrate for chromatography.

6.2.3 Identification

Identify the pyridoxine by comparison of the retention time of the individual peaks in the chromatograms obtained with the test sample solution, and with the standard test solution. Peak identification can also be performed by adding the pyridoxine standard substance to the sample test solution.

The separation and the quantification have proven to be satisfactory if following experimental conditions are followed (see also Figure A.1).

Stationary phase:	LiChrospher ® 60 RP C8 Select B, 5 µm, 250 mm x 4,0 mm;
Mobile phase:	according to 4.22;
Flow rate:	1 ml/min;
Injection volume:	30 µl;
Detection: Fluorometric:	Excitation: 290 nm; Emission: 395 nm.

6.2.4 Determination by HPLC

Inject the same appropriate volumes (up to 50 µl) of the standard solution as well as of the sample test solution into the HPLC-system. To carry out a determination by external calibration, integrate the peak areas or peak heights and compare the results with the corresponding values for the standard substance.

7 Calculation

Base the calculation on a calibration graph, or use the corresponding programs of the integrator, or use the following simplified procedure.

Calculate the mass fraction, w, of vitamin B_6 as pyridoxine in mg/100 g of the sample using Formula (4):

$$w = \frac{A_s \cdot \rho \cdot 100 \cdot 2}{A_{st} \cdot m \cdot 1000} \cdot 100 \cdot 0{,}822 \qquad (4)$$

where

A_s	is the peak area or peak height for pyridoxine obtained with the sample test solution, in units of area or height;
A_{st}	is the peak area or peak height for pyridoxine obtained with the standard test solution, in units of area or height;
ρ	is the mass concentration of pyridoxine hydrochloride in the standard test solution, in micrograms per millilitre;
m	is the sample mass, in gram;
100	is the total volume of the sample test solution, in millilitre;
2	is the factor of dilution of the reaction with sodium borohydride if acetic acid is added, otherwise the dilution factor is 1,9;
1 000	is the factor to convert microgram to milligram;
100	is the factor to calculate the content per 100 g;
0,822	is the factor to convert pyridoxine hydrochloride to pyridoxine.

Report the result for vitamin B_6 calculated as pyridoxine in mg/100 g.

8 Test report

The test report should comply with EN ISO/IEC 17025 [14] and shall contain at least the following data:

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

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

c) the date and time of sampling procedure (if known);

d) the date of receipt;

e) the date of test;

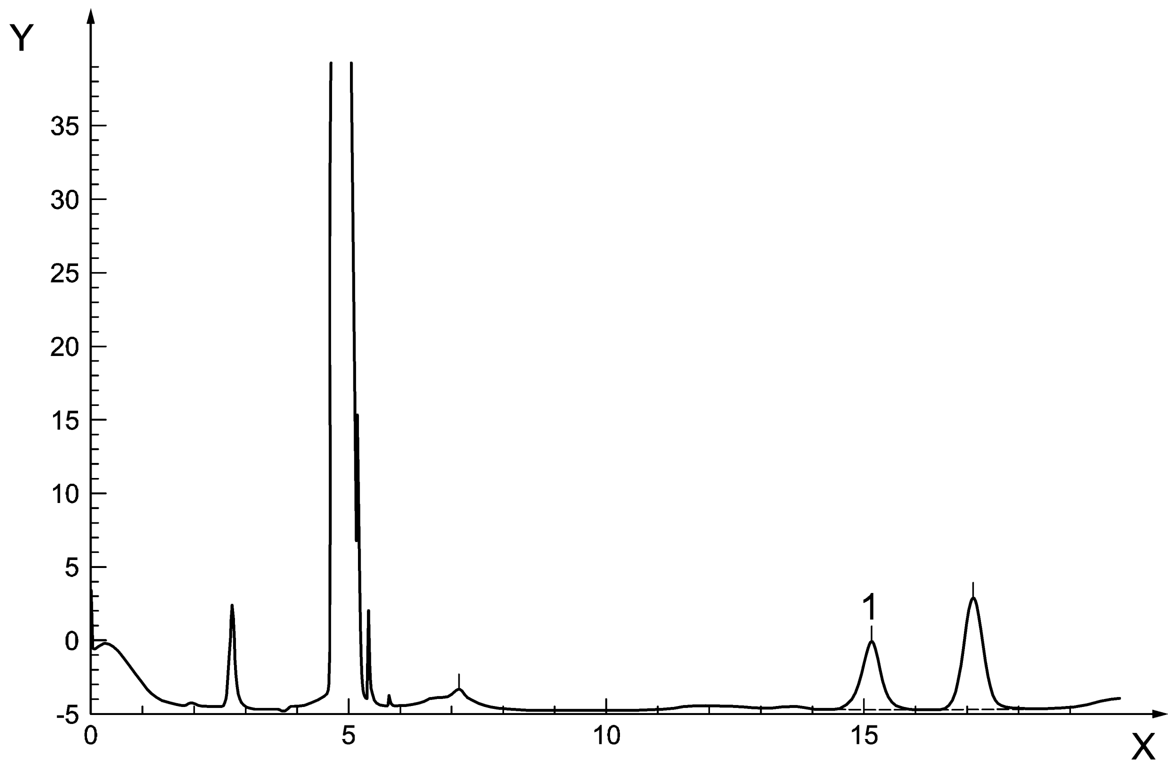
f) the results and the units in which the results have been expressed;

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

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

Annex A
(informative)

Example of a chromatogram



Key

Y	fluorescence
X	time (min)
1	pyridoxine

Stationary phase:	LiChrospher ® 60 RP C8 Select B, 5 µm, 250 mm x 4,0 mm
Mobile phase:	according to 4.22
Flow rate:	1 ml/min
Injection volume:	30 µl
Detection:	fluorometric: excitation: 290 nm; emission: 395 nm

Figure A.1 — Example for an HPLC quantification of pyridoxine in milk powder

Annex B
(informative)

Precision data

The existing data have been mainly obtained by using the method as outlined in Annex C without any acid hydrolysis. The results are not modified by the acid hydrolysis.

The precision data for the determination of vitamin B_6 in Table B.1 were established in an interlaboratory test according to ISO 5725 [10] carried out by DGCCRF (Direction Génerale de la Concurrence, de la Consommation et de le Repression des Fraudes). The collaborative study has been carried out by using the following analytical column: LiChrospher ® 60 RP 8 Select B, particle size of 5 µm, diameter of 4,0 mm and length of 250 mm.

The precision data for the determination of vitamin B_6 in Table B.2 were established in an interlaboratory test according to AOAC Guidelines for collaborative study procedures to validate characteristics of a method of analysis, see [11] carried out by Mann et al., see [7] and [8] and based on Bergaentzlé's method, see [3]. The collaborative study has been carried out using a Luna® 5 µm phenyl-hexyl HPLC column[4)].

Table B.1 — Precision data for the determination of vitamin B_6

Sample[a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Year of interlaboratory test	1993	1993	1993	1993	1993	1993	1993	1993
Number of laboratories	12	12	12	12	12	12	12	12
Number of laboratories retained after eliminating outliers	11	10	11	12	11	11	12	11
Number of results retained	32	29	31	36	33	33	35	33
Mean value, $\bar{x}$, mg/100 g	0,06	0,14	0,22	0,53	0,55	0,67	1,50	3,28
Repeatability standard deviation, s_r, mg/100 g	0,01	0,02	0,02	0,04	0,02	0,03	0,10	0,09
Repeatability relative standard deviation, RSD_r, %	18	13	10	8	4	4	6	3
Repeatability limit, r, [$r = 2,8 \times s_r$], mg/100 g	0,03	0,05	0,06	0,12	0,06	0,08	0,27	0,26
Reproducibility standard deviation, s_R, mg/100 g	0,02	0,05	0,07	0,14	0,07	0,08	0,18	0,43
Reproducibility relative standard deviation, RSD_R, %	30	35	30	26	13	12	12	13
Reproducibility limit, R, [$R = 2,8 \times s_R$], mg/100 g	0,05	0,14	0,19	0,39	0,20	0,23	0,51	1,22
Horrat values, according to [12]	1,7	2,3	2,1	2,1	1,1	1,1	1,1	1,4
[a] 1 Baby food, 2 Biscuit, 3 Cereal B, 4 Yeast, 5 Tube-feeding solution, 6 Chocolate powder, 7 Cereal A, 8 Powdered milk								

4) Luna ® 5 µm phenyl-hexyl HPLC column is an example of a suitable product available commercially. This information is given for the convenience of users and does not constitute an endorsement by CEN of the product named. Equivalent products can be used if they lead to equivalent results.

Table B.2 — Precision data for the determination of vitamin B_6 in reconstituted infant formula

Sample[a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Year of interlaboratory test	2003	2003	2003	2003	2003	2003	2003	2003
Number of laboratories	11	11	11	11	11	11	11	11
Number of laboratories retained after eliminating outliers	9	9	8	9	7	9	9	9
Number of results retained	18	18	16	18	14	18	18	18
Mean value, $\bar{x}$, mg/100 g	0,013	0,036	0,057	0,101	0,005	0,028	0,056	0,106
Repeatability standard deviation s_r, mg/100 g	0,001	0,001	0,001	0,004	0,001	0,002	0,003	0,004
Repeatability relative standard deviation, RSD_r, %	10	3,3	2,0	4,0	16,4	5,9	4,5	3,5
Repeatability limit r [r = 2,8 × s_r], mg/100 g	0,0028	0,0028	0,0028	0,0112	0,0028	0,0056	0,0084	0,0112
Reproducibility standard deviation s_R, mg/100 g	0,002	0,003	0,005	0,006	0,002	0,003	0,004	0,007
Reproducibility relative standard deviation, RSD_R, %	17,5	8,2	8,4	8,4	52,1	11,2	7,4	6,4
Reproducibility limit R [R = 2,8 × s_R], mg/100 g	0,0056	0,0084	0,014	0,0168	0,0056	0,0084	0,0112	0,0196
Horrat values [12]	0,81	0,44	0,48	0,53	2,06	0,58	0,42	0,43

[a] 1 Non-fortified milk based infant formula, 2 to 4 fortified milk based infant formula, 5 non-fortified soy based infant formula, 6 to 8 fortified soy based infant formula.

Annex C
(informative)

Sample treatment option without acid hydrolysis

Replace 6.2.1 to 6.2.2 of the main text by the following procedure:

Weigh an appropriate amount of the sample to the nearest mg, e.g. 2,5 g (if the vitamin B_6 content exceeds 2 µg/g) or 5,0 g (if the vitamin content is less than 2 µg/g) in a conical flask. Add 25 ml of sodium acetate solution 0,05 mol/l (4.14), 2,5 ml of glyoxylic acid 1 mol/l (4.19), 400 µl of ferrous sulfate solution (4.15) and 20 mg of acid phosphatase (4.1). Incubate the solution overnight at 37 °C with continuous agitating.

After cooling to room temperature dilute to 50 ml with water in a volumetric flask. Shake and filter. Mix 5,0 ml of filtrate and 4,5 ml of 0,1 mol/l sodium borohydride solution (4.18). Shake for at least for 3 min. Add 0,5 ml of glacial acetic acid (4.3). Shake for 1 min. Filter through a 0,45 µm filter. Use this filtrate for chromatography. At the calculation step in Formula (4), replace 100 by 50 (total volume of the sample test solution in ml).

Annex D
(informative)

Examples for molar absorption coefficients

Table D.1 — Examples for molar absorption coefficients (*ε*) of vitamin B_6 compounds [6], [13]

Compounds	Solvent	λ_{max} nm	*ε* $l\ mol^{-1}\ cm^{-1}$	*M* $g\ mol^{-1}$
Pyridoxine hydrochloride	0,1 mol/l HCl, pH ≈ 1	290	8 600	205,6
Pyridoxine hydrochloride	0,1 mol/l phosphate buffer, pH = 7	323,8	7 300	205,6
Pyridoxal hydrochloride	0,1 mol/l HCl, pH ≈ 1	288	8 960 (9 000)	203,6
Pyridoxal-5'-phosphate	0,1 mol/l phosphate buffer, pH = 7	388	5 020	247,1
Pyridoxamine dihydrochloride	0,1 mol/l HCl, pH ≈ 1	292	8 200	241,1
Pyridoxamine dihydrochloride	0,1 mol/l phosphate buffer, pH = 7	253	4 600	241,1
Pyridoxamine-5'-phosphate hydrochloride	0,1 mol/l phosphate buffer, pH = 7	326	8 370	241,1
Pyridoxal-5'-phosphate	0,1 mol/l HCl, pH ≈ 1	293	7 200	247,1

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