BRITISH STANDARD

BS EN 15652:2009

# Foodstuffs — Determination of niacin by HPLC

ICS 67.050



BSI British Standards

# National foreword

This British Standard is the UK implementation of EN 15652:2009.

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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EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

**EN 15652**

May 2009

ICS 67.050

English Version

# Foodstuffs - Determination of niacin by HPLC

Produits alimentaires - Dosage de la niacine par CLHP

Lebensmittel - Bestimmung von Niacin mit HPLC

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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EUROPÄISCHES KOMITEE FÜR NORMUNG

**Management Centre: Avenue Marnix 17, B-1000 Brussels**



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# Foreword

This document (EN 15652:2009) 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of an identical text or by endorsement, at the latest by November 2009, and conflicting national standards shall be withdrawn at the latest by November 2009.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 the mass fraction of niacin in foodstuffs by high performance liquid chromatography (HPLC) by three different ways of hydrolysis, acid hydrolysis (A), enzymatic hydrolysis (B) or acid/alkaline hydrolysis (C).

The method has been validated in interlaboratory tests on fortified and non-fortified samples such as breakfast cereal powder, chocolate cereals, cooked ham, green peas, lyophilized green peas with ham, lyophilized soup, nutritive orange juice, milk powder and wheat flour, at levels from 0,5 mg/100 g to 24 mg/100 g. For further information on the validation data, see Annex B.

A and B give similar results for niacin. In options A and B niacin is calculated as the sum of nicotinamide and nicotinic acid, and expressed as nicotinic acid [1]. Option C gives higher results than A and B for niacin with non-supplemented cereals, but similar results for other products. In option C, niacin is calculated and expressed as nicotinic acid after transformation of nicotinamide into nicotinic acid [2].

Option A is faster and cheaper than B and C.

Option B is used if an exact quantification of nicotinamide and nicotinic acid is needed. This cannot be done with option A, because there is a slight transformation of nicotinamide into nicotinic acid during the acid hydrolysis.

Option C quantifies total niacin. The alkaline hydrolysis is able to liberate other forms giving higher results for niacin, which in some foods such as maize and cereals are not normally biologically available, see [3], [4] and [5].

Information on a comparison between the three different ways of hydrolysis is given in Annex C.

# 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

Niacin vitamers are extracted from food by an acid (option A), an enzymatic (option B) or an acid/alkaline (option C) treatment and quantified by HPLC with a fluorimetric detection after a post-column derivatization with UV irradiation, see [1] and [2]. For option A and option B, niacin is determined as the sum of nicotinamide and nicotinic acid. Niacin is expressed as nicotinic acid after correction of the molecular weights. For option C, niacin is determined and expressed as nicotinic acid. The alkaline treatment transforms all nicotinamide into nicotinic acid.

# 4 Reagents

## 4.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of at least grade 1 according to EN ISO 3696:1995.

## 4.2 Chemicals and solutions

**4.2.1 Sodium acetate,** mass fraction $w(C_2H_3NaO_2) \geq 99$ %

**4.2.2 Potassium monohydrogen phosphate,** $w(K_2HPO_4) \geq 99,5$ %

**4.2.3 Potassium dihydrogen phosphate**, $w(KH_2PO_4) \geq 99,5$ %

**4.2.4 Non stabilized hydrogen peroxyde solution,** $w(H_2O_2) = 30$ %

**4.2.5 Copper sulfate,** $w(Cu(II)SO_4 \cdot 5H_2O) \geq 99$ %

**4.2.6 Acetic acid,** $w(CH_3COOH) \geq 99,8$ %

**4.2.7 Concentrated hydrochloric acid solution (option A and C)**, $w(HCl) = 37,0$ %

**4.2.8 NADase from *Neurospora crassa* (option B), enzyme activity 0,55 U/mg of protein.**

Store below 0 °C.

NOTE For the interlaboratory study, NADase from *Neurospora crassa* from Sigma Chemicals with reference N 9629, lyophilised powder, 0,5 U/mg to 3,0 U/mg protein (biuret) has been used[1].

**4.2.9 Acetic acid solution,** substance concentration $c(CH_3COOH) = 5$ mol/l

**4.2.10 Sodium acetate solution,** $c(C_2H_3NaO_2) = 2,5$ mol/l

**4.2.11 Copper sulfate solution,** $c(Cu(II)SO_4 \cdot 5H_2O) = 0,005$ mol/l

**4.2.12 Sodium acetate solution (option B),** $c(C_2H_3NaO_2) = 0,05$ mol/l, pH = 4,5

Dissolve 4,10 g of sodium acetate (4.2.1) in 900 ml of water. Adjust the solution to pH = 4,5 with acid acetic (4.2.6), and then dilute to 1000 ml with water.

**4.2.13 Phosphate buffer solution (option B),** $c(K_2HPO_4) = 0,05$ mol/l and $c(KH_2PO_4) = 0,05$ mol/l, pH = 6,8

Mix 1 part per volume of $K_2HPO_4$ solution (0,05 mol/l) and 1 part per volume of $KH_2PO_4$ solution (0,05 mol/l). Adjust pH to 6,8 with sodium acetate solution (4.2.10) if necessary.

**4.2.14 NADase solution (option B)**

Dissolve 2,9 mg of NADase (4.2.8) in 5 ml of phosphate buffer (4.2.13). This solution is stable for 1 week at -18 °C.

**4.2.15 Hydrochloric acid solution (options A and C),** $c(HCl) = 0,1$ mol/l

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.

#### 4.2.16 HPLC mobile phase

Dissolve 4,77 g of potassium dihydrogen phosphate (4.2.3) in 400 ml of water. Add 3,8 ml of hydrogen peroxide solution (4.2.4) and 0,5 ml of copper sulfate solution (4.2.11). Dilute to 500 ml. The pH is about 4,5. Filter through a membrane filter (5.7). This solution is stable for one day.

#### 4.2.17 Sodium hydroxide (option C), $w(NaOH) \geq 99$ %

#### 4.2.18 Sodium hydroxide solution (option C), $c(NaOH) = 5$ mol/l

Dissolve 20 g of sodium hydroxide (4.2.17) in 80 ml of water. After cooling dilute to 100 ml.

#### 4.2.19 Hydrochloric acid solution (option C), $w(HCl) = 3,7$ %

Dilute 5 ml of the concentrated hydrochloric acid solution (4.2.7) to 50 ml with water.

### 4.3 Standard substances

#### 4.3.1 General

Nicotinic acid and nicotinamide can be obtained from various suppliers. The purity may vary and it is therefore necessary to determine the concentration of the calibration solution by a spectrometric determination (see 4.4.3).

#### 4.3.2 Nicotinic acid, $w(C_6H_5NO_2) \geq 99,5$ %

#### 4.3.3 Nicotinamide (options A and B), $w(C_6H_6N_2O) \geq 99,5$ %

### 4.4 Stock solutions

#### 4.4.1 Nicotinic acid stock solution, mass concentration $\rho(C_6H_5NO_2) = 1$ mg/ml

Dissolve an amount of the nicotinic acid standard substance (4.3.2), e.g. approximately 100 mg to the nearest 1 mg in 100 ml of water. This solution is stable for 1 week at -18 °C.

#### 4.4.2 Nicotinamide stock solution, (options A and B), $\rho(C_6H_6N_2O) = 1$ mg/ml

Dissolve an amount of the nicotinamide standard substance (4.3.3), e.g. approximately 100 mg to the nearest 1 mg in 100 ml of water. This solution is stable for 1 week at -18 °C.

#### 4.4.3 Concentration tests

##### 4.4.3.1 Nicotinic acid solution, $\rho(C_6H_5NO_2) = 1$ mg/ml

Dilute 1 ml of the nicotinic acid stock solution (4.4.1) in 100 ml of hydrochloric acid solution (4.2.15) and measure the absorbance at 260 nm in a 1 cm cell using a UV spectrometer (5.2) against hydrochloric acid solution (4.2.15) as reference. Calculate the mass concentration, $\rho$, in milligram per millilitre of the stock solution, using Equation (1):

$$\rho = \frac{A_{260} \times 1000}{420} \qquad (1)$$

where

$A_{260}$ is the absorbance value of the solution at 260 nm;

420 is the $E_{1cm}^{1\%}$ value for nicotinic acid in 0,1 mol/l HCl, see [6].

#### 4.4.3.2 Nicotinamide solution, $\rho(C_6H_6N_2O)$ = 1 mg/ml

Dilute 1 ml of the nicotinamide stock solution (4.4.2) in 100 ml of hydrochloric acid solution (4.2.15) and measure the absorbance at 260 nm in a 1 cm cell using a UV spectrometer (5.2) against hydrochloric acid solution (4.2.15) as reference. Calculate the mass concentration, $\rho$, in milligram per millilitre of the stock solution, using Equation (2):

$$\rho = \frac{A_{260} \times 1000}{410} \qquad (2)$$

where

$A_{260}$ is the absorbance value of the solution at 260 nm;

410 is the $E_{1cm}^{1\%}$ value for nicotinamide in 0,1 mol/l HCl, see [6].

### 4.5 Nicotinic acid and nicotinamide standard solutions, $\rho(C_6H_5N0_2)$ = $\rho(C_6H_6N_2O)$ = 0,05 µg/ml to 5 µg/ml

Prepare e.g. a first solution with 1 ml of each stock solution (4.4.1) or (4.4.2) in 100 ml of water. From this solution prepare four standard solutions (0,5 ml, 2,5 ml, 10 ml and 50 ml) in 100 ml of water. These solutions are stable for one day at room temperature.

## 5 Apparatus

### 5.1 General

Usual laboratory apparatus and glassware, and the following.

### 5.2 UV spectrometer

Capable of measurement of absorbance at defined wavelenghts

### 5.3 Oven, capable of maintaining a temperature of 37 °C

### 5.4 Autoclave, capable of maintaining a temperature of 120 °C

### 5.5 HPLC system

Consisting of a pump, sample injecting device, fluorescence detector with excitation and emission wavelengths set at 322 nm and 380 nm, and an evaluation system such as an integrator.

### 5.6 Analytical reverse phase separating column, e.g. LiChrospher® 60 RP-18 Select B endcapped[2]

The column, with the following characteristics, shall ensure a baseline resolution of the analytes concerned:

a) a length of 25 cm;

b) an inner diameter of 4,0 mm;

c) a particle size of 5 µm.

Other particle sizes or column dimensions than specified in this European Standard may be used. Separation parameters shall be adapted to such other materials to guarantee equivalent results.

### 5.7 Filter device

Membrane filter with a pore size of for example 0,45 µm.

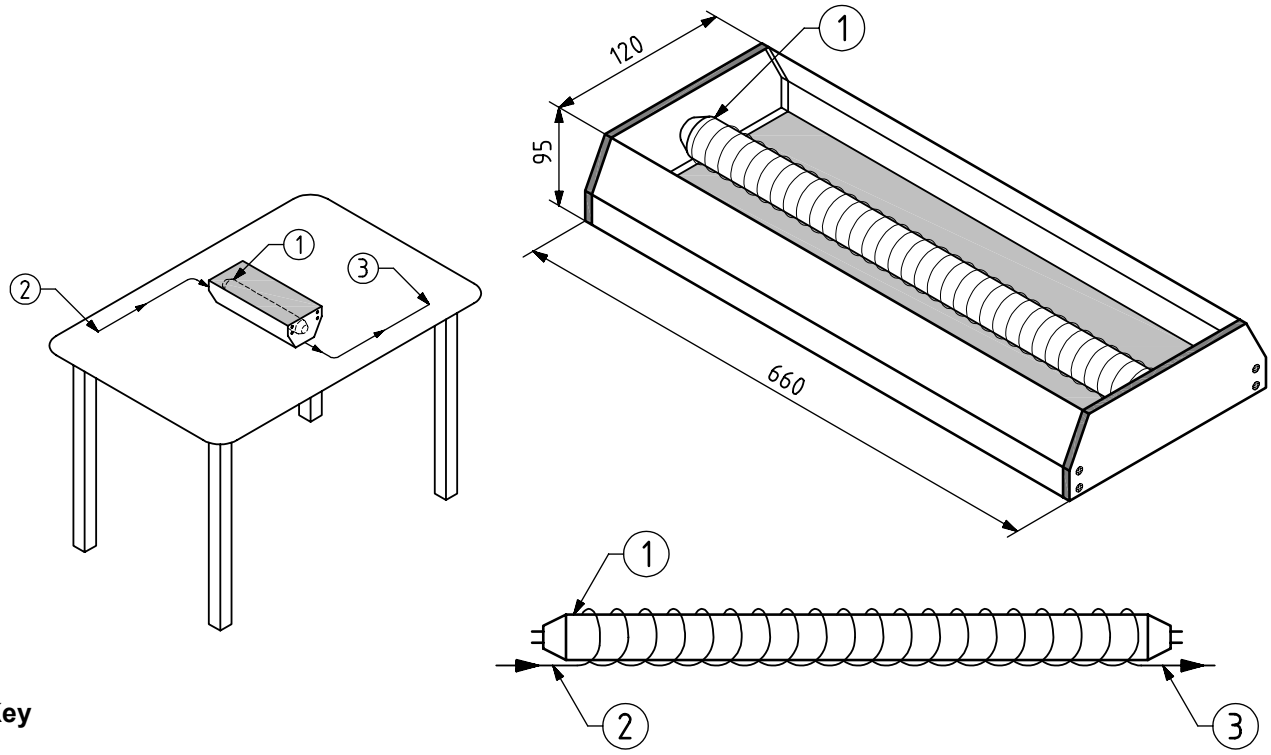
### 5.8 Post-column derivatization tube and UV lamp

A polytetrafluoroethylene (PTFE) tube (length of 5 m, inner diameter of 0,5 mm, external diameter of 1,6 mm) surrounding a UV black-light-blue (BLB) lamp with low-pressure tube (VL-120 BLB, 20 W, 365 nm, intensity is 55 µW/cm$^2$ from Vilber Lourmat)[2)], see Figure 1 and Figure 2 and also [7].

**WARNING 1 — Harmful UV light could come out of the metal box containing the lamp.**

**WARNING 2 — If bubble formation occurs in the tube due to overheating, the tube should be efficiently cooled by air circulation, for example by lifting the box.**

2 LiChrospher® 60 RP-18 Select B endcapped and VL-120 BLB are examples of suitable products available commercially. This information is given for the convenience of users of ths 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.

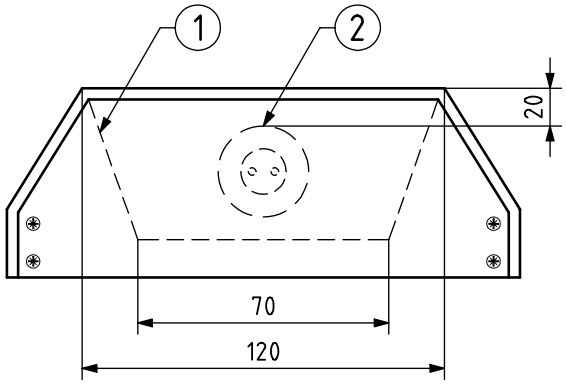



**Key**

1 lamp tube
2 from column
3 to detector

**Figure 1 — Schematic representation and dimensions (mm) of the lamp, lamp housing (in upside down position) and placement of the lamp housing on bench (in operating position)**

Dimensions in millimetres




**Key**

1 reflector
2 lamp tube

**Figure 2 — Cross section of the lamp housing (in upside down position) with tube lamp and dimensions**

# 6 Procedure

## 6.1 Sample preparation

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 Extraction

### 6.2.1 Extraction option A, acid hydrolysis

Similar results are obtained as option B for niacin, but with a slight transformation of nicotinamide to nicotinic acid during the hydrolysis step.

Weigh an appropriate amount of the test sample to the nearest mg, e.g. 1 g to 5 g in a conical flask. Add 50 ml of the hydrochloric acid solution (4.2.15). Let the conical flask in a boiling water bath for 1 h. After cooling add sodium acetate (4.2.10) solution to reach a pH of 4,5. Transfer to a volumetric flask. Make up to 100 ml with water. Shake the solution and filter through a paper filter. Filter again through a membrane filter (5.7) before injection.

NOTE Filtering of the mobile phase as well as the test sample solution through a membrane filter prior to use or injection will increase the longevity of the column.

### 6.2.2 Extraction option B, enzymatic hydrolysis

Similar results are obtained as option A for niacin. Option B allows an exact quantification of nicotinamide and nicotinic acid.

Weigh an appropriate amount of the test sample to the nearest mg, e.g. 1 g to 5 g in a conical flask. Add 50 ml of the sodium acetate solution (4.2.12). Add 200 µl of the NADase solution (4.2.14). Let the conical flask in an oven (5.3) at 37 °C with stirring for 18 h. After cooling, transfer to a volumetric flask. Make up to 100 ml with water. Shake the solution and filter through a paper filter. Filter again through a membrane filter (5.7) before injection.

### 6.2.3 Extraction option C, acid and alkaline hydrolysis

Higher results are obtained for non-supplemented cereal products than with options A and B. Similar results are obtained for other food samples. Option C gives a total niacin quantification as nicotinic acid after transformation of nicotinamide into nicotinic acid by the alkaline step.

Weigh an appropriate amount of the test sample to the nearest mg, e.g. 1 g to 5 g in a conical flask. Add 70 ml of hydrochloric acid solution (4.2.15). Let the conical flask in a boiling water bath for 1 h. After cooling add 1 ml to 2 ml of the sodium hydroxide solution (4.2.18) to reach a pH of 4,5. Transfer the solution to a volumetric flask. Make up to 100 ml with water. Filter through a paper filter. Transfer 50 ml of the filtrate in a conical flask. Add 10 ml of the sodium hydroxide solution (4.2.18). Autoclave at 120 °C for 1 h. After cooling, adjust the pH to 4,5 by adding hydrochloric acid. Start with the concentrated hydrochloric acid solution (4.2.7) and go on with the diluted hydrochloric acid solution (4.2.19). Transfer to a volumetric flask. Make up to 100 ml with water. Filter through a paper filter. Filter again through a membrane filter (5.7) before injection.

## 6.3 Chromatography

Inject equal volumes of the standard solutions and the sample test solutions into the HPLC system. Identify the analytes by comparison of the retention time of the individual peaks in the chromatograms obtained with the sample test solution, and with the standard solution. Adding the standard substances to the sample test solution can also perform peak identification.

The separation and the quantification was proven to be satisfactory if the following experimental conditions are followed:

Stationary phase: Lichrospher® 60 RP Select B, particle size of 5 µm, diameter of 4,0 mm, length of 250 mm;

Mobile phase: Phosphate buffer ($c$ = 0,07 mol/l), hydrogen peroxyde ($c$ = 0,075 mol/l), copper sulfate ($c$ = 5 x $10^{-6}$ mol/l);

Flow rate: 1 ml/min;

Injection volume: 30 µl;

Detection: Fluorimetric: excitation: 322 nm; emission: 380 nm.

# 7 Calculation

## 7.1 General

To carry out a determination by external calibration, use a calibration curve, or integrate the peak areas or determine the peak heights and compare the results with the corresponding values for the standard solution with the nearest peak area or height. Check the linearity of the calibration.

## 7.2 Calculation for option A or B

Calculate the mass fraction, $w$, of nicotinic acid in mg/100 g of the sample, using Equation (3):

$$w = \frac{A_{TS} \times \rho \times V_e}{A_{ST} \times 10 \times m_s} \quad (3)$$

where

$A_{TS}$ is the peak area or peak height for the derivate obtained with the sample test solution, in units of area or height;

$\rho$ is the mass concentration of nicotinic acid in the standard solution (4.5), in microgram per millilitre;

$V_e$ is the volume of sample test solution (6.2.1 or 6.2.2) in millilitre;

$A_{ST}$ is the peak area or peak height for the derivate obtained with the standard solution, in units of area or height;

$m_s$ is the sample mass, in g.

Calculate the mass fraction, $w'$, of nicotinamide in mg/100 g of the sample, using Equation (4):

$$w' = \frac{A'_{TS} \times \rho' \times V_e}{A'_{ST} \times 10 \times m_s} \quad (4)$$

where

$A'_{TS}$ is the peak area or peak height for the derivate obtained with the sample test solution, in units of area or height;

$\rho'$ is the mass concentration of nicotinamide in the standard solution (4.5), in microgram per millilitre;

$V_e$ is the volume of sample test solution (6.2.1 or 6.2.2), in millilitre;

$A'_{ST}$ is the peak area or peak height for the derivate obtained with the standard solution, in units of area or height;

$m_s$ is the sample mass, in g.

Report the result for niacin in ($w$ + 1,008 $w'$) expressed in nicotinic acid in mg/100 g.

### 7.3 Calculation for option C

Calculate the mass fraction, $w$, of nicotinic acid in mg/100 g of the sample, using Equation (5):

$$w = \frac{A_{TS} \times \rho \times V_e \times 2}{A_{ST} \times 10 \times m_s} \quad (5)$$

where:

$A_{TS}$ is the peak area or peak height for the derivate obtained with the sample test solution, in units of area or height;

$\rho$ is the mass concentration of nicotinic acid in the standard solution (4.5), in microgram per millilitre;

$V_e$ is the volume of sample test solution (6.2.3), in millilitre;

$A_{ST}$ is the peak area or peak height for the derivate obtained with the standard solution, in units of area or height;

$m_s$ is the sample mass, in g.

## 8 Precision

### 8.1 General

The precision data of this HPLC method for the determination of niacin with options A and B were established according to ISO 5725-2 [10] in 2002 by an international collaborative study organized by AéRIAL (CRT: Centre de Ressources technologiques) and the CGd'UMA (Commission Générale d'Unification des Méthodes d'Analyses), see [1]. The study provided the statistical information given in Table B.1 and Table B.2 of Annex B.

The precision data for the determination of niacin with option C were established according to ISO 5725-2 [10] in 1999 by a French collaborative study organized by CGd'UMA, see [2]. The study provided the statistical information given in Table B.3 of Annex B.

### 8.2 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 niacin with option A, acid hydrolysis, are:

Milk powder: $\bar{x}$ = 16,66 mg/100 g $r$ = 1,31 mg/100 g

| | | |
|---|---|---|
| Chocolate cereals: | $\bar{x}$ = 21,03 mg/100 g | *r* = 0,68 mg/100 g |
| Ham: | $\bar{x}$ = 16,91 mg/100 g | *r* = 0,53 mg/100 g |
| Wheat flour: | $\bar{x}$ = 0,72 mg/100 g | *r* = 0,079 mg/100 g |
| Green peas: | $\bar{x}$ = 5,91 mg/100 g | *r* = 0,93 mg/100 g |

The values for niacin with option B, enzymatic hydrolysis, are:

| | | |
|---|---|---|
| Milk powder: | $\bar{x}$ = 17,08 mg/100 g | *r* = 1,39 mg/100 g |
| Chocolate cereals: | $\bar{x}$ = 21,24 mg/100 g | *r* = 1,75 mg/100 g |
| Ham: | $\bar{x}$ = 17,29 mg/100 g | *r* = 0,70 mg/100 g |
| Wheat flour: | $\bar{x}$ = 0,54 mg/100 g | *r* = 0,040 mg/100 g |
| Green peas: | $\bar{x}$ = 5,79 mg/100 g | *r* = 0,33 mg/100 g |

The values for niacin with option C, acid and alkaline hydrolysis, are:

| | | |
|---|---|---|
| Breakfast cereals: | $\bar{x}$ = 23,92 mg/100 g | *r* = 2,29 mg/100 g |
| Chocolate cereals: | $\bar{x}$ = 16,98 mg/100 g | *r* = 2,24 mg/100 g |
| Milk powder: | $\bar{x}$ = 5,66 mg/100 g | *r* = 0,92 mg/100 g |
| Fruit juice: | $\bar{x}$ = 4,31 mg/100 g | *r* = 0,49 mg/100 g |
| Lyophilized green peas with ham: | $\bar{x}$ = 12,89 mg/100 g | *r* = 1,78 mg/100 g |
| Lyophilized soup: | $\bar{x}$ = 11,06 mg/100 g | *r* = 0,53 mg/100 g |

### 8.3 Reproducibility

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

The values for niacin with option A, acid hydrolysis, are:

| | | |
|---|---|---|
| Milk powder: | $\bar{x}$ = 16,66 mg/100 g | *R* = 2,04 mg/100 g |
| Chocolate cereals: | $\bar{x}$ = 21,03 mg/100 g | *R* = 2,55 mg/100 g |
| Ham: | $\bar{x}$ = 16,91 mg/100 g | *R* = 1,75 mg/100 g |
| Wheat flour: | $\bar{x}$ = 0,72 mg/100 g | *R* = 0,59 mg/100 g |
| Green peas: | $\bar{x}$ = 5,91 mg/100 g | *R* = 3,68 mg/100 g |

The values for niacin with option B, enzymatic hydrolysis, are:

| | | |
|---|---|---|
| Milk powder: | $\bar{x}$ = 17,08 mg/100 g | *R* = 2,07 mg/100 g |
| Chocolate cereals: | $\bar{x}$ = 21,24 mg/100 g | *R* = 3,08 mg/100 g |
| Ham: | $\bar{x}$ = 17,29 mg/100 g | *R* = 3,76 mg/100 g |
| Wheat flour: | $\bar{x}$ = 0,54 mg/100 g | *R* = 0,91 mg/100 g |
| Green peas: | $\bar{x}$ = 5,79 mg/100 g | *R* = 1,96 mg/100 g |

The values for niacin with option C, acid and alkaline hydrolysis, are:

| | | |
|---|---|---|
| Breakfast cereals: | $\bar{x}$ = 23,92 mg/100 g | *R* = 11,65 mg/100 g |
| Chocolate cereals: | $\bar{x}$ = 16,98 mg/100 g | *R* = 7,02 mg/100 g |

| | | |
|---|---|---|
| Milk powder: | $\bar{x}$ = 5,66 mg/100 g | *R* = 2,82 mg/100 g |
| Fruit juice: | $\bar{x}$ = 4,31 mg/100 g | *R* = 0,54 mg/100 g |
| Lyophilized green peas with ham: | $\bar{x}$ = 12,89 mg/100 g | *R* = 6,61 mg/100 g |
| Lyophilized soup: | $\bar{x}$ = 11,06 mg/100 g | *R* = 3,51 mg/100 g |

## 9 Test report

The test report shall contain at least the following data:

a) all information necessary for the identification of the sample (kind of sample, origin of sample, designation);

b) a reference to this European Standard and the extraction option chosen;

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

d) the date of sample receipt;

e) the date of test;

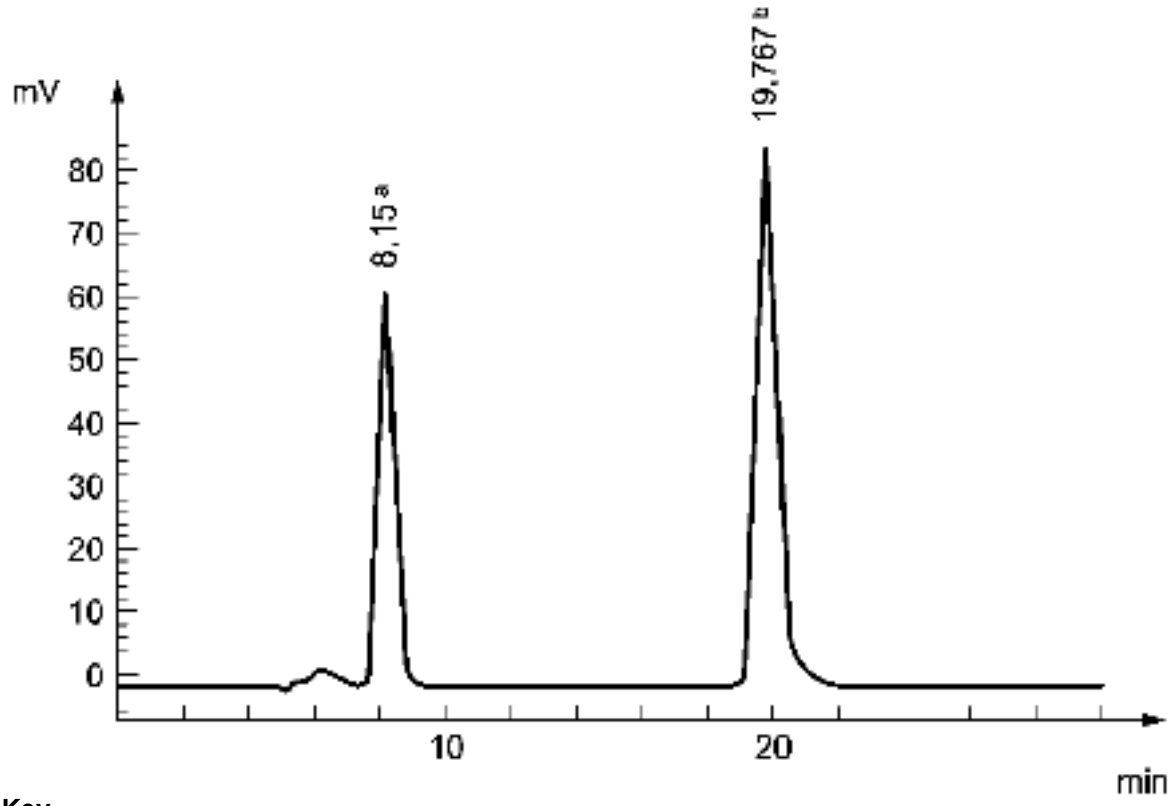
f) the test results and the units in which they have been expressed;

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 effected the results.

# Annex A
(informative)

# Typical chromatogram




**Key**
a nicotinic acid
b nicotinamide

**Figure A.1 — Example of a HPLC separation of nicotinic acid and nicotinamide standards using post-column derivatization**

Experimental conditions for Figure A.1 are:

Stationary phase: Lichrospher® 60 RP Select B, particle size of 5 µm, diameter of 4,0 mm, length of 250 mm;

Mobile phase: Phosphate buffer ($c$ = 0,07 mol/l), hydrogen peroxyde ($c$ = 0,075 mol/l), copper sulfate ($c = 5 \times 10^{-6}$ mol/l);

Flow rate: 1 ml/min;

Injection volume: 30 µl;

Detection: Fluorimetric: excitation: 322 nm; emission: 380 nm.

# Annex B
(informative)

# Precision data for acid-, enzymatic- and acid/alkaline hydrolysis

The precision data for the determination of niacin with acid- (option A) and enzymatic- (option B) hydrolysis in Table B.1 and Table B.2 were established according to ISO 5725-2 [10] in 2002 by an international collaborative study organized by AéRIAL (CRT: Centre de Ressources technologiques) and the CGd'UMA (Commission Générale d'Unification des Méthodes d'Analyses), see [1].

The precision data for the determination of niacin with option C in Table B.3 were established according to ISO 5725-2 [10] in 1999 by a French collaborative study organized by CGd'UMA, see [2].

**Table B.1 — Precision data with acid hydrolysis**

| **Samples** | **Milk powder (fortified)** | **Chocolate cereals (fortified)** | **Ham (not fortified)** | **Wheat flour (not fortified)** | **Green peas (not fortified)** |
|---|---|---|---|---|---|
| Year of interlaboratory test | 2002 | 2002 | 2002 | 2002 | 2002 |
| Number of laboratories | 12 | 12 | 12 | 12 | 12 |
| Number of samples (duplicates) | 2 | 2 | 2 | 2 | 2 |
| Number of laboratories retained after eliminating outliers | 11 | 10 | 10 | 12 | 12 |
| Number of outliers | 1 | 2 | 2 | 0 | 0 |
| Number of accepted results | 22 | 20 | 20 | 24 | 24 |
| Mean value, $\bar{x}$, mg/100 g | 16,66 | 21,03 | 16,91 | 0,72 | 5,91 |
| Repeatability standard deviation $s_r$, mg/100 g | 0,46 | 0,24 | 0,19 | 0,028 | 0,33 |
| Repeatability relative standard deviation $RSD_r$, % | 2,8 | 1,1 | 1,1 | 3,9 | 5,6 |
| Repeatability limit $r$ [$r = 2{,}83 \times s_r$], mg/100 g | 1,31 | 0,68 | 0,53 | 0,079 | 0,93 |
| Reproducibility standard deviation $s_R$, mg/100 g | 0,72 | 0,90 | 0,62 | 0,21 | 1,30 |
| Reproducibility relative standard deviation $RSD_R$, % | 4,3 | 4,3 | 3,7 | 29,2 | 22,0 |
| Reproducibility limit $R$ [$R = 2{,}83 \times s_R$], mg/100 g | 2,04 | 2,55 | 1,75 | 0,59 | 3,68 |
| Horrat values [8] | 0,6 | 0,6 | 0,5 | 2,5 | 2,5 |

**Table B.2 — Precision data with enzymatic hydrolysis**

| **Samples** | **Milk powder (fortified)** | **Chocolate cereals (fortified)** | **Ham (not fortified)** | **Wheat flour (not fortified)** | **Green peas (not fortified)** |
|---|---|---|---|---|---|
| Year of interlaboratory test | 2002 | 2002 | 2002 | 2002 | 2002 |
| Number of laboratories | 12 | 12 | 12 | 12 | 12 |
| Number of samples (duplicates) | 2 | 2 | 2 | 2 | 2 |
| Number of laboratories retained after eliminating outliers | 11 | 10 | 11 | 12 | 12 |
| Number of outliers | 1 | 2 | 1 | 0 | 0 |
| Number of accepted results | 22 | 20 | 22 | 24 | 24 |
| Mean value, $\bar{x}$, mg/100 g | 17,08 | 21,24 | 17,29 | 0,54 | 5,79 |
| Repeatability standard deviation $s_r$, mg/100 g | 0,49 | 0,62 | 0,25 | 0,014 | 0,12 |
| Repeatability relative standard deviation $RSD_r$, % | 2,9 | 2,9 | 1,4 | 2,6 | 2,0 |
| Repeatability limit $r$ [$r = 2{,}83 \times s_r$], mg/100 g | 1,39 | 1,75 | 0,70 | 0,040 | 0,33 |
| Reproducibility standard deviation $s_R$, mg/100 g | 0,73 | 1,09 | 1,33 | 0,32 | 0,69 |
| Reproducibility relative standard deviation $RSD_R$, % | 4,3 | 5,1 | 7,7 | 59,2 | 11,9 |
| Reproducibility limit $R$ [$R = 2{,}83 \times s_R$], mg/100 g | 2,07 | 3,08 | 3,76 | 0,91 | 1,96 |
| Horrat values [8] | 0,6 | 0,7 | 1,0 | 4,8 | 1,4 |

**Table B.3 — Precision data with acid/alkaline hydrolysis**

| Samples | Breakfast cereals (fortified) | Chocolate cereals (not fortified) | Milk powder (not fortified) | Fruit juice (fortified) | Lyophilized green peas with ham (not fortified) | Lyophilized soup (not fortified) |
|---|---|---|---|---|---|---|
| Year of interlaboratory test | 1999 | 1999 | 1999 | 1999 | 1999 | 1999 |
| Number of laboratories | 11 | 11 | 11 | 10 | 10 | 10 |
| Number of samples (duplicates) | 2 | 2 | 2 | 2 | 2 | 2 |
| Number of laboratories retained after eliminating outliers | 10 | 11 | 9 | 8 | 10 | 10 |
| Number of outliers | 1 | 0 | 2 | 3 | 0 | 0 |
| Number of accepted results | 20 | 22 | 18 | 16 | 20 | 20 |
| Mean value, $\bar{x}$, mg/100 g | 23,92 | 16,98 | 5,66 | 4,31 | 12,89 | 11,06 |
| Repeatability standard deviation $s_r$, mg/100 g | 0,81 | 0,79 | 0,32 | 0,17 | 0,63 | 0,19 |
| Repeatability relative standard deviation $RSD_r$, % | 3,4 | 4,7 | 5,7 | 4,0 | 4,9 | 1,7 |
| Repeatability limit $r$ [$r = 2{,}83 \times s_r$], mg/100 g | 2,29 | 2,24 | 0,92 | 0,49 | 1,78 | 0,53 |
| Reproducibility standard deviation $s_R$, mg/100 g | 4,11 | 2,48 | 0,99 | 0,19 | 2,34 | 1,24 |
| Reproducibility relative standard deviation $RSD_R$, % | 17,2 | 14,6 | 17,6 | 4,5 | 18,1 | 11,2 |
| Reproducibility limit $R$ [$R = 2{,}83 \times s_R$], mg/100 g | 11,65 | 7,02 | 2,82 | 0,54 | 6,61 | 3,51 |
| Horrat values [8] | 2,5 | 2,0 | 2,0 | 0,5 | 2,4 | 1,4 |

# Annex C
(informative)

# Comparison between three different ways of hydrolysis

Table C.1 gives a comparison of values of total niacin obtained in different samples after three different ways of hydrolysis, acid hydrolysis (A), enzymatic hydrolysis (B) and acid/alkaline hydrolysis (C). Further information on this comparison is given in [9].

**Table C.1 — Comparison of total niacin with three different ways of hydrolysis**

| Samples | A (in mg/100 g) | B (in mg/100 g) | C (in mg/100 g) |
|---|---|---|---|
| **Non-supplemented food** | | | |
| Beef liver | 51,6 | 57,8 | 43,0 |
| Ham | 16,9 | 17,1 | |
| Dried egg yolk | 0,05 | 0,05 | 0,26 |
| Green Peas 1 | 7,2 | 6,4 | 8,7 |
| Green Peas 2 | 5,9 | 5,7 | |
| Wheat flour 1 | 0,8 | 0,5 | 2,4 |
| Wheat flour 2 | 0,7 | 0,5 | |
| Dried brewer's yeast | 13,9 | 13,4 | 17,7 |
| Rice 1 | 1,3 | 1,3 | 3,6 |
| Rice 2 | 0,4 | 0,3 | 3,2 |
| High fiber content product | 1,1 | 1,1 | 3,7 |
| **Supplemented food** | | | |
| Chocolate powder 1 | 2,1 | 2,7 | 1,2 |
| Chocolate powder 2 | 0,4 | 0,8 | 0,7 |
| Milk powder 1 | 8,2 | 7,9 | 9,3 |
| Milk powder 2 | 16,7 | 17,1 | |
| Milk powder 3 | 4,2 | 4,3 | 3,5 |
| Fruit Juice | 0,3 | 0,3 | 0,3 |
| Breakfast Cereals 1 | 20,0 | 19,6 | 19,0 |
| Breakfast Cereals 2 | 21,0 | 21,2 | |
| Breakfast Cereals 3 | 21,9 | 20,7 | 19,9 |
| Breakfast Cereals 4 | 12,5 | 12,3 | 12,0 |
| Breakfast Cereals 5 | 16,1 | 18,1 | 16,5 |
| Breakfast Cereals 6 | 15,4 | 15,6 | 15,2 |
| Flour for infant | 4,0 | 4,3 | 4,7 |
| Dietetic bars | 15,4 | 16,4 | 15,4 |
| High protein content powder 1 | 9,4 | 11,4 | 9,7 |

**Table C.1 (continued)**

| | | | |
|---|---|---|---|
| High protein content powder 2 | 10,7 | 10,3 | 9,0 |
| High protein content powder 3 | 10,5 | 10,0 | 10,8 |
| Meal substitute | 15,7 | 15,4 | 13,5 |
| Food supplement 1 | 2 004 | 2 197 | 2 203 |
| Food supplement 2 | 487 | 505 | 493 |
| Food supplement 3 | 2 236 | 2 498 | 2 199 |
| Food supplement 4 | 1 044 | 1 201 | 1 112 |
| Food supplement 5 | 47,5 | 47,4 | 45,7 |

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[10] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

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