# SLOVENSKI STANDARD
# SIST EN 15652:2009

**01-september-2009**

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**Živila - Določevanje niacina s HPLC**

Foodstuffs - Determination of niacin by HPLC

Lebensmittel - Bestimmung von Niacin mit HPLC

Produits alimentaires - Dosage de la niacine par CLHP



**Ta slovenski standard je istoveten z:** **EN 15652:2009**

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# Foodstuffs - Determination of niacin by HPLC

Produits alimentaires - Dosage de la niacine par CLHP

Lebensmittel - Bestimmung von Niacin mit HPLC

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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of an identical text or by endorsement, at the latest by November 2009, and conflicting national standards shall be withdrawn at the latest by November 2009.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 $E^{1\%}_{1cm}$ 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 $E^{1\%}_{1cm}$ 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

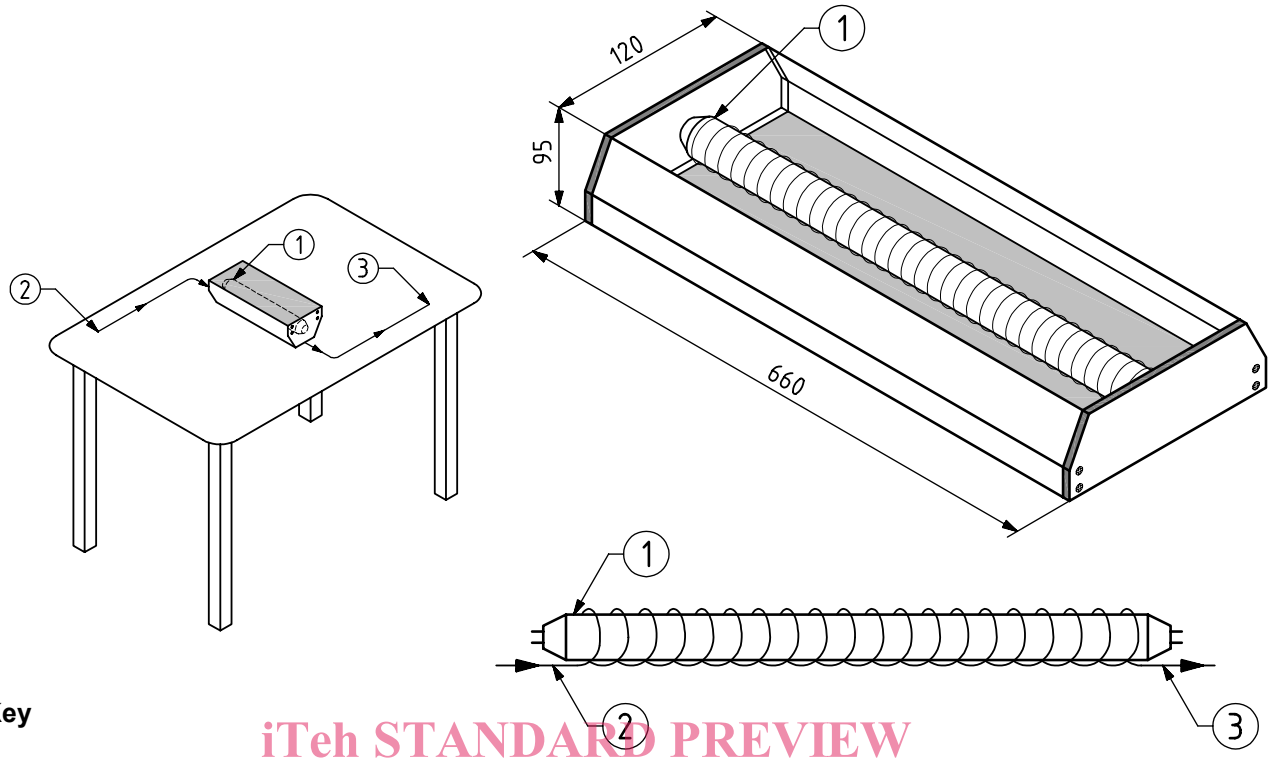
A polytetrafluoroethylene (PTFE) tube (length of 5 m, inner diameter of 0,5 mm, external diameter of 1,6 mm) surrounding a UV black-light-blue (BLB) lamp with low-pressure tube (VL-120 BLB, 20 W, 365 nm, intensity is 55 µW/cm$^2$ from Vilber Lourmat)[2)], see Figure 1 and Figure 2 and also [7].

**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.



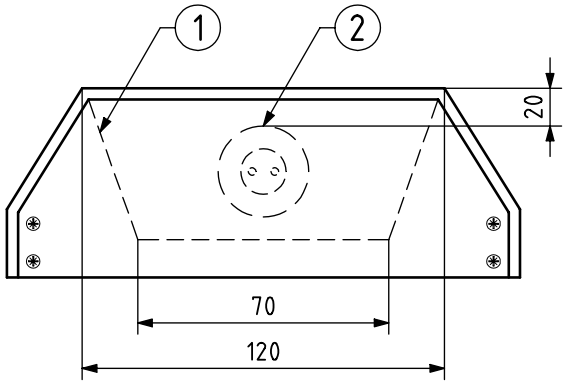

**Key**

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**