# SLOVENSKI STANDARD
# SIST EN 14131:2003

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**Živila - Določevanje folata z mikrobiološko metodo**

Foodstuffs - Determination of folate by microbiological assay

Lebensmittel - Mikrobiologische Bestimmung von Folat

Produits alimentaires - Détermination des folates par essai microbiologique



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# Foodstuffs - Determination of folate by microbiological assay

Produits alimentaires - Détermination des folates par essai microbiologique

Lebensmittel - Mikrobiologische Bestimmung von Folat

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

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# Contents

# Foreword

This document (EN 14131:2003) 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of an identical text or by endorsement, at the latest by December 2003, and conflicting national standards shall be withdrawn at the latest by December 2003.

Annexes A and B are informative.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Luxembourg, Malta, Netherlands, Norway, Portugal, Slovakia, Spain, Sweden, Switzerland and the United Kingdom.

## 1 Scope

This European Standard specifies a microbiological method for the determination of the total folate content of foodstuffs by turbidimetric detection of the growth of the microorganism *Lactobacillus casei,* subsp. *rhamnosus* (ATCC 7469).

The method allows for the determination of folates in foodstuffs, including naturally occurring folates and added folic acid (pteroylglutamic acid).



## 2 Normative references



This European Standard 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 Standard 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, *Water for analytical laboratory use — Specifications and test methods (ISO 3696:1987).*

## 3 Principle

Samples suspended in phosphate buffer are heated to enable extraction of folates. Protease and α-amylase treatment may be used to further digest the food matrix. Naturally occurring folylpolyglutamates are hydrolysed with γ-glutamyl hydrolase (EC 3.4.19.9) [1] to folylmono- or folyldi-glutamates.

Extracted folates are diluted with basal medium containing all required growth nutrients except folate. The growth response of *Lactobacillus casei*, subsp. *rhamnosus* (ATCC 7469) to extracted folates is followed turbidimetrically and is compared to the growth response to calibrant solutions with known concentration.

The method allows for the optional use of a semi-automated liquid-handling system and of a microplate or test tubes for incubation of the microorganism.

# 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. The water used for reagent preparation shall be glass distilled.

## 4.2 Solvents and chemicals

### 4.2.1 Glycerol, $w(C_3H_8O_3)$ = 80 %

Mix 120 ml of glycerol with 30 ml of glass-distilled water.

### 4.2.2 1-Octanol, $C_8H_{18}O$

### 4.2.3 Toluene, $C_7H_8$

### 4.2.4 2-Mercaptoethanol, $c(C_2H_6OS)$ = 0,1 mol/l

Add 70 µl of 2-mercaptoethanol to 10 ml of water.

### 4.2.5 Sodium ascorbate, $C_6H_7O_6Na$



Sodium ascorbate is used as a reagent in several solutions specified in this draft standard. Ascorbic acid may equally well be used, but procedures for pH adjustment may need to be modified.



### 4.2.6 Hydrochloric acid, $c$(HCl) = 1 mol/l



### 4.2.7 Sodium hydroxide, $w$(NaOH) = 40 %

Dissolve 400 g of sodium hydroxide in water and dilute to 1 l.

### 4.2.8 Ammonium sulfate, $H_8N_2O_4S$

### 4.2.9 Sodium phosphate, monobasic, anhydrous, $NaH_2PO_4$

The amounts of monobasic sodium phosphate used for buffer preparation (4.3) have been calculated for the anhydrous substance. The mono- or dihydrated substance may also be used, with the procedures adjusted accordingly.

### 4.2.10 2-(N-Cyclohexylamino)ethanesulfonic acid (CHES), $C_8H_{17}NO_3S$

### 4.2.11 N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), $C_8H_{18}N_2O_4S$

### 4.2.12 Carbon powder, acid-washed

### 4.2.13 Saline, sterile

Dissolve 9 g of sodium chloride in 1000 ml of water. Dispense 10-ml portions into 20 mm x 150-mm test tubes. Cap tubes and heat at + 121 °C for 15 min. Cool and store refrigerated.

**4.2.14 Folic acid-free basal medium solution,** double strength

For each 100 ml needed, suspend the recommended amount of basal medium (Bacto Folic Acid Casei Medium or equivalent[1)]) in 100 ml of glass-distilled water. Add 0,050 g of sodium ascorbate (4.2.5) and heat to boiling for 1 min to 2 min. Allow cooling to room temperature and adjust pH to 6,1 ± 0,1.

**4.2.15 Folic acid standard substance**

Folic acid can be obtained from various suppliers and may contain up to 8 % water. The purity of the folic acid standard may vary and it is therefore necessary to determine the concentration of the calibration solution by UV absorption measurement (see procedure for standardisation in 6.4.2).

## 4.3 Buffers

**4.3.1 Phosphate buffer,** pH 5,0 ($c$ = 0,002 mol/l)

Dissolve 0,24 g of sodium phosphate, monobasic (4.2.9) in 900 ml of water. Adjust pH to 5,0 ± 0,1 with sodium hydroxide (4.2.7) and dilute to 1000 ml with water.

**4.3.2 Phosphate buffer,** pH 7,0 ($c$ = 0,1 mol/l)

Dissolve 12,0 g of sodium phosphate, monobasic (4.2.9) in 900 ml of water. Adjust pH to 7,0 ± 0,1 with sodium hydroxide (4.2.7) and dilute to 1000 ml with water.



**4.3.3 Phosphate buffer,** pH 5,0 ($c$ = 0,1 mol/l) with 2-mercaptoethanol ($c$ = 10 mmol/l)

Dissolve 12,0 g of sodium phosphate, monobasic (4.2.9) in 900 ml of water. Adjust pH to 5,0 ± 0,1, add 0,70 ml of 2-mercaptoethanol (4.2.4) and dilute to 1000 ml with water.



**4.3.4 Phosphate buffer,** pH 4,5 ($c$ = 0,1 mol/l) with ascorbate (1 %)

Dissolve 12,0 g of sodium phosphate, monobasic (4.2.9) and 10 g of sodium ascorbate (4.2.5) in 900 ml of water. Adjust pH to 4,5 ± 0,1 with sodium hydroxide (4.2.7) and dilute to 1000 ml with water. Prepare fresh on day of use.

**4.3.5 Phosphate buffer,** pH 6,1 ($c$ = 0,1 mol/l) with ascorbate (1 %)

Dissolve 12,0 g of sodium phosphate, monobasic (4.2.9) and 10 g of sodium ascorbate (4.2.5) in 900 ml of water. Adjust pH to 6,1 ± 0,1 with sodium hydroxide (4.2.7) and dilute to 1000 ml with water. Prepare fresh on day of use.

**4.3.6 Phosphate buffer,** pH 7,8 ($c$ = 0,1 mol/l) with ascorbate (1 %)

Dissolve 12,0 g of sodium phosphate, monobasic (4.2.9) and 10 g of sodium ascorbate (4.2.5) in 900 ml of water. Adjust pH to 7,8 ± 0,1 with sodium hydroxide (4.2.7) and dilute to 1000 ml with water. Prepare fresh on day of use.

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1) Bacto Folic Acid Casei Medium is the trade name of a product supplied by Difco. 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.

**4.3.7 CHES/HEPES buffer,** pH 7,85 (*c* = 0,05 mol/l) with ascorbate and 2-mercaptoethanol (4.2.4) for the dialysis of rat blood plasma.

Dissolve 23,8 g of HEPES (4.2.11), 20,7 g of CHES (4.2.10), 40 g of sodium ascorbate (4.2.5) and 1,4 ml of 2-mercaptoethanol (4.2.4) in 1900 ml of water. Adjust pH to 7,85 ± 0,1 with sodium hydroxide (4.2.7) and dilute to 2000 ml with water. Add 4 g of acid-washed carbon powder (4.2.12). Prepare fresh on day of use.

## 4.4 Enzymes

### 4.4.1 Additional enzyme treatment (optional)

Additional incubation with protease and/or α-amylase may be used to enhance extractability of the folates. Procedures for additional enzyme treatment are further discussed in Annex A.

### 4.4.2 γ-Glutamyl hydrolase

#### 4.4.2.1 General

Deconjugase activity is provided by γ-glutamyl hydrolase (EC 3.4.19.9) 0 from one of several sources. An example describing the preparation of γ-glutamyl hydrolase from hog kidney is given below (4.4.2.2). Procedures for the preparation of γ-glutamyl hydrolase from optional sources are given in Annex A. The appropriateness of the chosen enzyme preparation shall be checked with a suitable technique.

NOTE A yeast powder, lyophilised pig's liver (e.g. BCR CRM 487 [2]), or to a limited extent pteroyl-triglutamic acid can be appropriate samples to use for the checking of the enzyme preparation.



#### 4.4.2.2 γ-Glutamyl hydrolase source: hog kidney [3]

Homogenise 250 g of fresh hog kidney at + 2 ºC in 750 ml of phosphate buffer with 2-mercaptoethanol (4.3.3). Centrifuge at + 2 ºC (18000 *g*, 20 min). Incubate supernatant at + 50 ºC for 2 h with gentle agitation and repeat centrifugation. Fractionate the supernatant by precipitation with saturated ammonium sulfate (4.2.8). Collect the fraction precipitated between 50 % and 75 % saturation with ammonium sulfate. Suspend precipitate in a minimal volume of phosphate buffer (4.3.1). Dialyse against the same buffer 2 x 24 h and centrifuge (18000 *g*, 20 min). Transfer 1-ml aliquots to vials and lyophilise.



## 4.5 Inoculum

### 4.5.1 Test organism

Lyophilised culture of *Lactobacillus casei* subsp. *rhamnosus* (ATCC 7469)[2)].

### 4.5.2 Culture medium

Dilute 50 ml of double-strength folic acid-free basal medium solution (4.2.14) to 100 ml with glass-distilled water. Add 0,5 ml of diluted folic acid stock solution (6.4.4), mix and sterile filter or heat at + 121 ºC for 15 min and rapidly cool to room temperature.

### 4.5.3 Cryoprotected inoculum

Aseptically, add 1 ml of the prepared culture medium (4.5.2) to the lyophilised culture (4.5.1) and transfer 0,15 ml of the resulting suspension to the culture medium according to 4.5.2. Incubate at + 37 ºC for 18 h.

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2) Distributors include National Collection of Industrial and Marine Bacteria Ltd (Aberdeen, UK) and Culture Collection, University of Göteborg (Gothenburg, Sweden). This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of the distributors named.

Heat 150 ml of glycerol (4.2.1) at + 121 ºC for 15 min and cool in ice bath. Cool incubated bacterial culture in ice bath and add 100 ml sterilised and cooled glycerol. Mix gently. Dispense 2-ml aliquots into sterile vials. Store at – 20 ºC for up to three months or at – 70 ºC for up to six months.

NOTE It is essential to maintain aseptic conditions throughout the whole process.

#### 4.5.4 Working inoculum

##### 4.5.4.1 Working inoculum for tube cultures

Dilute 2 ml cryoprotected inoculum (4.5.3) to 50 ml with sterile saline (4.2.13). Vortex mix.

##### 4.5.4.2 Working inoculum for microplate cultures (optional)

Add 5 ml of sterile saline (4.2.13) to 2 ml cryoprotected inoculum (4.5.3). Vortex mix.

#### 4.5.5 **Inoculated folic acid-free basal medium solution,** for microplate assay (optional).

Add 1 µl working inoculum (4.5.4.2) per 1 ml folic acid-free basal medium solution (4.2.14). Mix thoroughly.

## 5 Apparatus

Usual laboratory apparatus, glassware and, in particular, the following:



**5.1 Centrifuge, cooled,** for preparation of hog kidney conjugase (4.4.2.2), suitable for 18 000 *g*.

**5.2 Heating device,** Autoclave or equivalent



**5.3 Incubator,** or water-bath for incubation at (+37 ±0,2) ºC



**5.4 Spectrometer/nephelometer,** for measurement of turbidity of folate extracts after incubation in test tubes

**5.5 Test tube racks with cover,** to hold test tubes during heating and incubation

**5.6 Dialysis tube (optional),** with molecular mass cut-off of 12000 to 14000, for the preparation of rat plasma conjugase (Annex A).

**5.7 Liquid-handling system (optional),** for automated assay-tube reading

**5.8 Microplate reader (optional),** for measurement of turbidity after incubation in microplates

**5.9 Flat-bottomed 96-well microplates, sterile (optional)**

# 6 Procedure

## 6.1 General

Folates are sensitive to UV light and oxidation. Perform all operations away from natural and strong fluorescent light. Use amber glassware where possible.

Carry out the determination in duplicate as single determinations on two different occasions.

## 6.2 Preparation of test sample

The test sample shall be homogeneous. Coarse material shall be rendered homogeneous using an appropriate mill and/or blender. Care shall be taken to avoid exposure to high temperatures during this process. Homogenised test samples shall be stored in air-tight containers, free from exposure to light.

## 6.3 Preparation of sample solution

### 6.3.1 General

Depending on the sample type the preparation of the test sample solution may include protease and/or α-amylase treatment to hydrolyse proteins and carbohydrates, thus facilitating folate extraction. The sample extract is treated with γ-glutamyl hydrolase in order to hydrolyse naturally occurring folylpolyglutamates to folylmono- or folyldi-glutamates. This method uses γ-glutamyl hydrolase from hog kidney, but other sources may be used provided that sufficient enzyme activity is ensured. Procedures for additional enzyme treatment and the use of γ-glutamyl hydrolase from other sources are further described in Annex A.



### 6.3.2 Extraction



Accurately weigh a suitable amount of the test sample (corresponding to 2 g to 2,5 g dry weight) into a 100-ml volumetric flask. Add 20 ml of phosphate buffer with ascorbate pH 6,1 (4.3.5) and mix thoroughly. Add 30 ml of water and 0,1 ml to 1 ml of 1-octanol (4.2.2). Cover flask and heat at 100 °C to 121 °C for 15 min. Cool, and dilute to 100 ml with phosphate buffer with ascorbate pH 6,1 (4.3.5).

### 6.3.3 γ-Glutamyl hydrolase treatment (hog kidney)

Reconstitute the vial of lyophilised hog kidney γ-glutamyl hydrolase with 1,1 ml water and store in ice. Carefully mix in a 10-ml volumetric flask: 1 ml of sample extract (6.3.2), 3,5 ml of phosphate buffer with ascorbate pH 4,5 (4.3.4) and 0,5 ml of reconstituted γ-glutamyl hydrolase. Incubate at + 37 °C for 3 h. Inactivate enzyme by heating at 100 °C for 3 min. Cool, and add to volume with phosphate buffer with ascorbate pH 4,5 (4.3.4). Centrifuge (1000 g, 10 min) to remove precipitated protein. Store supernatant at – 18 °C for subsequent analysis.

## 6.4 Calibration

### 6.4.1 Folic acid stock solution (100 µg/ml)

Accurately weigh 50 mg of folic acid (4.2.15) that has been dried to constant weight and dissolve in phosphate buffer (4.3.2) in a 500-ml volumetric flask. Dilute to volume with phosphate buffer. Top with toluene to keep surface covered.

### 6.4.2 Concentration test of folic acid stock solution

Transfer 10 ml of stock solution (6.4.1) to a 100-ml volumetric flask and dilute to volume with phosphate buffer pH 7,0 (4.3.2). Measure the absorbance of the solution at 282 nm using phosphate buffer as a blank. Calculate the folic acid concentration ($\rho$) in gram per litre according equation (1):

$$\rho = \frac{A \cdot M}{\varepsilon \cdot b} \cdot V$$

where:

*A* is the absorbance value of the solution at 282 nm;

*M* is the molar mass of folic acid, i.e. 441,4 g $mol^{-1}$;

*ε* is the molar absorption coefficient of folic acid [4] at 282 nm, i.e. 27600 l $mol^{-1}$ $cm^{-1}$;

*b* is the absorption path length in cm;

*V* is the dilution factor (if any).

#### 6.4.3 Folic acid standard solution (1 µg/ml)

Add 5 ml of stock solution (6.4.1) to 475 ml with water and adjust pH to 7,5 with sodium hydroxide (4.2.7). Dilute to 500 ml with water. Prepare fresh on day of use. Further dilutions may be prepared to suit the assay format used.

#### 6.4.4 Diluted folic acid standard solution (100 ng/ml) for use in inoculum.

Add 10 ml of standard solution (6.4.3) to approximately 60 ml of water and adjust pH to 7,5 with sodium hydroxide (4.2.7). Dilute to 100 ml with water. Prepare fresh on the day of use.



### 6.5 Determination



#### 6.5.1 General

Determine the folic acid content in the enzyme preparations used and compensate accordingly.

#### 6.5.2 Treatment of standard solution (1 µg/ml)

Treat 1 ml of standard solution (6.4.3) as a test sample and subject it to the extraction procedure and the relevant enzyme treatment as described in 6.3. The resulting folic acid concentration is nominally 10 ng/ml.

#### 6.5.3 Dilution of extracts and treated standard solution

##### 6.5.3.1 General

The dilution steps in 6.5.3.2, 6.5.3.3, 6.5.3.4 apply to the standard test tube procedure. For assay in the 96-well microplate format, proceed to 6.5.4.3.

The dilution step may be adjusted to compensate for low or high folate concentration levels.

##### 6.5.3.2 Calibration solution (0,3 ng/ml)

Pipette 7,5 ml of treated standard solution (6.5.2) into a 250-ml volumetric flask. Add 7,5 ml of phosphate buffer (4.3.5) and dilute to 250 ml with water.

##### 6.5.3.3 Secondary calibration solutions (0,2 ng/ml and 0,4 ng/ml) (optional)

Pipette 5 ml and 10 ml of treated standard solution (6.5.2) into 250-ml volumetric flasks. Add an equal volume of phosphate buffer pH 6,1 (4.3.5) and dilute to 250 ml with water.