

**Foodstuffs – Determination of folate by
microbiological assay**

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Foodstuffs - Determination of folate by microbiological assay

Produits alimentaires - Détermination des folates par essai
microbiologique

Lebensmittel - Mikrobiologische Bestimmung von Folat

This European Standard was approved by CEN on 21 April 2003.

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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of an identical text or by endorsement, at the latest by December 2003, and conflicting national standards shall be withdrawn at the latest by December 2003.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.

EN 14131:2003 (E)**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**, $w(\text{C}_3\text{H}_8\text{O}_3) = 80 \%$

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

4.2.2 1-Octanol, $\text{C}_8\text{H}_{18}\text{O}$ **4.2.3 Toluene**, C_7H_8 **4.2.4 2-Mercaptoethanol**, $c(\text{C}_2\text{H}_6\text{OS}) = 0,1 \text{ mol/l}$

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

4.2.5 Sodium ascorbate, $\text{C}_6\text{H}_7\text{O}_6\text{Na}$

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, $c(\text{HCl}) = 1 \text{ mol/l}$ **4.2.7 Sodium hydroxide**, $w(\text{NaOH}) = 40 \%$

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

4.2.8 Ammonium sulfate, $\text{H}_8\text{N}_2\text{O}_4\text{S}$ **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), $\text{C}_8\text{H}_{17}\text{NO}_3\text{S}$ **4.2.11 N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES)**, $\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$ **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 equivalent¹⁾) 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 $6,1 \pm 0,1$.

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 $5,0 \pm 0,1$ 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 $7,0 \pm 0,1$ 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 $5,0 \pm 0,1$, 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 $4,5 \pm 0,1$ 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 $6,1 \pm 0,1$ 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 $7,8 \pm 0,1$ with sodium hydroxide (4.2.7) and dilute to 1000 ml with water. Prepare fresh on day of use.

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.

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4.3.7 CHES/HEPES buffer, pH 7,85 ($c = 0,05 \text{ mol/l}$) 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 $7,85 \pm 0,1$ 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 $+ 2 \text{ }^\circ\text{C}$ in 750 ml of phosphate buffer with 2-mercaptoethanol (4.3.3). Centrifuge at $+ 2 \text{ }^\circ\text{C}$ (18000 g , 20 min). Incubate supernatant at $+ 50 \text{ }^\circ\text{C}$ 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 $+ 121 \text{ }^\circ\text{C}$ 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 $+ 37 \text{ }^\circ\text{C}$ for 18 h.

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.