# भारतीय मानक

***Indian Standard***

***Determination of Total Folates in Foods***

***using LC-MS/MS***

 BIS 2024

भारतीय मानक ब्यरू ो

BUREAU OF INDIAN STANDARDS

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Test Methods for Food Products Sectional Committee, FAD 28

**FOREWORD**

This Indian Standard (First Revision) was adopted by the Bureau of Indian Standards, after the draft finalized by the Test Methods for Food Products Sectional Committee had been approved by the Agricultural and Food Products Division Council.

Vitamin B9, or folate, is crucial for DNA and RNA synthesis, cell division, and amino acid metabolism. Its active form, 5-methyltetrahydrofolate (5-MTHF), is vital for converting homocysteine to methionine, impacting protein synthesis and neurotransmitter function. Adequate folate is essential for fetal development, reducing the risk of neural tube defects.

This standard was originally published in 1976. While bringing out first revision of this standard, new auxiliary food products have been added which may be used for palate clearing. The standard has been brought out in the latest style and format of Indian Standard, and references to Indian Standards, wherever applicable, have been updated.

The composition of the Committee responsible for the formulation of this standard is given in Annex A.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated expressing the result of a test or analysis, shall be rounded off in accordance with IS 2: 2022 ‘Rules for rounding off numerical values (*second revision*)’. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

**DETERMINATION OF TOTAL FOLATES**

**IN FOODS USING LC-MS/MS**

## **SCOPE**

This document specifies a method for the quantitative determination of total folates that is folic acid and 5-methyl tetra hydro folic acid in foods including cereals and pulses, fruits and vegetables and nut and nut products.

1. **REFERENCES**

There are no normative references in this document.

1. **TERMINOLOGY**

For the purpose of this standard, the definitions given shall apply.

1. **PRINCIPLE**

Powder samples were reconstituted by dissolving 25 g powder sample and 50 mg α-amylase in 200 g warm water (40°C). Samples were digested at 40°C for 15 min followed by dilution with 40 mL buffer [2% ascorbic acid, 0.1% dithiothreitol (DTT), pH 4.5] and heating at 90°C for 30 min with stirring. Sample was then digested with protease solution (4 mg/mL) at 37°C for 30 min and transferred to a 100 mL volumetric flask with water. After filtration and addition of internal standard (IS), the filtrate was loaded on a strong anion exchange (SAX) cartridge, eluted, and evaporated at 50°C under nitrogen flow. Extracts were then reconstituted in 1.5 mL reconstitution solution (H2O, 1% ascorbic acid, 0.5% DTT) and filtered through a 0.22 µm membrane into an amber LC vial for UHPLC-MS/MS analysis.

1. **REAGENTS AND MATERIALS**

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled or demineralized water or water of equivalent purity.

* 1. **L-Ascorbic acid,** Sigma (St. Louis, MO) A4544, or equivalent
	2. **Ammonium acetate p.a,** Merck (Darmstadt, Germany), or equivalent
	3. **DTT,** VWR (Radnor, PA), or equivalent
	4. **Disodium hydrogen phosphate powder,** VWR, or equivalent
	5. **α-Amylase from porcine pancreas,** Type VI, >10 units/mg (Sigma A3176), or equivalent
	6. **α-Amylase from Bacillus subtilis**, approximately 50 units/mg (Fluka 10070; Buchs, Switzerland), or equivalent
	7. **Protease from Streptomyces griseus,** Type IV, >3.5 units/mg (Sigma P5147), or equivalent
	8. **Folic acid,** Schirck Laboratories (Jona, Switzerland) 59-30-3, or equivalent
	9. **(6R, S)-5-Me THF acid calcium salt,** Schirck Laboratories 151533-22-1, or equivalent
	10. **[13C5]-Folic acid,** Merck, or equivalent
	11. **[13C5] -(6S)-5-Me THF calcium salt,** Merck, or equivalent
	12. **Formic acid p.a,** Merck, or equivalent
	13. **Acetic acid glacial p.a,** Merck, or equivalent
	14. **Sodium hydroxide solution,** 1 M (Merck), or equivalent
	15. **Hydrochloric acid,** 1 M (Merck), optional
	16. **Hydrochloric acid, 37% p.a,** (Merck), or equivalent
	17. **Ortho-phosphoric acid**, 85% (Merck), or equivalent
	18. **Ethanol,** HPLC grade (Merck), or equivalent
	19. **Methanol,** HPLC grade (Merck), or equivalent
	20. **Isopropanol,** LC-MS grade (Merck), or equivalent
	21. **Acetonitrile,** LC-MS grade (Merck), or equivalent
	22. **Acetonitrile,** HPLC grade (Sigma 348)
1. **STANDARD AND SOLUTION PREPARATION**

### Mobile Phases and Prepared Solutions

* + 1. **Mobile Phase A-** Acetic acid 0.5% (v/v) in water. Into a 1000 mL volumetric flask, add 5.00 mL acetic acid. Add about 800 mL water. Mix well. Make up to volume with water. This solution remains stable for 1 week at room temperature.
		2. **Mobile Phase B** Acetonitrile.
		3. **Needle wash solvent-** Water–acetonitrile–isopropanol (5+2+3) + 2% (v/v) formic acid. Into a 1000 mL bottle with cap, mix 500 mL water, 200 mL acetonitrile, and 300 mL isopropanol. Add 18 mL formic acid. Mix well. This solution remains stable for 1 month at room temperature. Note: Needle wash solvent is instrument dependent. Solution to minimize carryover should be studied on each analytical system.
		4. **Extraction buffer-** Sodium phosphate buffer 100 mmol/L, ascorbic acid 2% (w/v), DTT 0.1% (w/v), pH 4.5. Into a 1000 mL beaker, weigh 14.20 g disodium hydrogen phosphate (Na2HPO4), 20.0 g ascorbic acid, and 1.0 g DTT. Add about 800 mL water, dissolve, and adjust to pH 4.5 with ortho-phosphoric acid 85%. Transfer into a 1000 mL volumetric flask and make up to volume with water. This solution remains stable for 2 weeks at 4°C.
		5. **Protease solution-** 4 mg/mL in water. Into a 100 mL volumetric flask, weigh 400 mg protease. Dissolve and make up to volume with water. Prepare this solution fresh on the day of use.
		6. **SPE eluting solution-** Acetonitrile–extraction buffer–acetic acid (6+3+1). Into a 250 mL bottle with cap, mix 150 mL acetonitrile, 75 mL extraction buffer, and 25 mL acetic acid using a measuring cylinder. This solution remains stable for 2 weeks at 4°C.
		7. **Dissolution solution A-** Sodium hydroxide 0.1 mol/L containing about 50 mL water, mix 5.0 mL sodium hydroxide solution 1 mol/L and 20 mL ethanol. Make up to volume with water. This solution remains stable for 2 weeks at 4°C.
		8. **Dissolution solution B-** Ammonium acetate 10 mmol/L, ascorbic acid 10% (w/v), DTT 2% (w/v)–methanol (1 + 3). Into a 50 mL beaker, weigh 38.5 mg ammonium acetate, 5.0 g ascorbic acid, and 1.0 g DTT. Add about 40 mL water, dissolve, and make up to volume with water. Mix 50 mL of this solution with 150 mL methanol. This solution remains stable for 2 weeks at 4°C.
		9. **Dissolution solution C-** Ascorbic acid 1% (w/v), DTT 0.5% (w/v). Into a 1000 mL volumetric flask, weigh 10.0 g ascorbic acid and 5.0 g DTT. Add about 800 mL water, dissolve, and make up to volume with water. This solution remains stable for 2 weeks at 4°C.

###  Stable Isotope Labelled Compounds, Individual, Internal Standard Stock Solutions

* + 1. **Folic acid stock standard solution,**

About 100 µg/mL. Into a 50 mL amber glass volumetric flask, weigh 5.00 ± 0.20 mg folic acid and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution A (6.1.7). Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

* + 1. **5-Me THF stock standard (approximately 100 µg/mL),**

Intoa 50 mL amber glass volumetric flask, weigh 5.00 ± 0.20 mg 5-Me THF acid calcium salt and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution B (6.1.8). Store in aliquots flushed with N2. This solution remains stable for 5months at –20°C.

* + 1. **Intermediate Solution**
		2. **Standard Mix 1 (intermediate solution, 5000 ng/mL),**

#### Into a 10 mL amber glass volumetric flask, transfer by pipetting the calculated amount of folic acid stock solution and the calculated amount of 5-Me THF (free form) stock solution to obtain an exact final concentration of folic acid and 5-Me THF in its free form of 500 ng/mL. Make up to volume with dissolution solution C (6.1.9). Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

#### 6.2.5 Standard Mix 2 (intermediate solution, 75 ng/mL),

Into a 10 mL amber glass volumetric flask, transfer by pipetting 150 µL of standard Mix 1. Make up to volume with dissolution solution C (6.1.9) Store in aliquots flushed with N2. This solution remains stable for 3 months at –20°C.

* 1. **Stock Standard Solution**

#### 6.3.1 [13C5]-Folic acid stock solution (approximately200 µg/mL), Into a 10 mL amber glass volumetric flask, weigh 2.00 ± 0.20 mg [13C5]-folic acid and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution A. Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

#### 6.3.2 [13C5] -(6S)-5-Me THF IS stock solution (approximately200 µg/mL)-Into a 10 mL amber glass volumetric flask, weigh 2.00 ± 0.20 mg [13C5] -(6S)-5-Me THF calcium salt and record the mass to 0.01 mg. Dissolve and make up to volume with dissolution solution B. Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

#### Working Standard Solution Preparation

#### IS mix working solution (5000 ng/mL), Into a 10 mL amber glass volumetric flask, transfer by pipetting the calculated amount of folic acid IS stock solution and the calculated amount of 5-Me THF IS (free form) stock solution to obtain an exact final concentration of folic acid and 5-Me THF IS in its free form of 500 ng/mL. Make up to volume with dissolution solution C (6.1.9). Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

#### Working standards, Working standard solutions, 1 to 400 ng/mL. Into separate 5 mL amber glass volumetric flasks, transfer by pipetting the appropriate volume of standard Mix 1 or standard Mix 2 and IS mix working solution. Make up to volume with dissolution solution C (6.1.9) The final concentration of folic acid or 5-Me THF in the working standard solution ranges from 1 to 400 ng/mL with an IS concentration of 50 ng/mL.

#### APPARATUS

* 1. **Waters Column,** UHPLC HSS T3, 1.8 µm; 2.1×150 mm (Waters Corp., Milford, MA) or equivalent.
	2. **Liquid chromatograph,** Agilent 1290 Infinity (Agilent Technologies, Santa Clara, CA) or equivalent.
	3. **Detector,** Agilent 6460 MS in positive electrospray ionization (ESI+) mode operating at unit resolution, or equivalent.
	4. **Amber glassware,** Standard laboratory Class A.
	5. **Micro pipet**, Adjustable (volumes from 2 to 20 µL) and disposable tips.
	6. **Micro pipet,** Adjustable (volumes from 10 to 100 µL) and disposable tips.
	7. **Micro pipet,** Adjustable (volumes from 100 to 1000 µL) and disposable tips.
	8. **Multi pette® plus**, Eppendorf (Hamburg, Germany), or equivalent.
	9. **Analytical balance,** Precision 0.1 mg.
	10. **Homogenizer,** Polytron 3100 (Kinematica, Lucerne, Switzerland), or equivalent.
	11. **pH meter,** Mettler-Toledo (Columbus, OH), or equivalent.
	12. **Water bath (up to 90°C),** With magnetic stirrers (Labotech; DWB 16) or equivalent.
	13. **Folded filters,** S&S 597½ (diameter 185 mm; Whatman, Piscataway, NJ), or equivalent.
	14. **Solid phase extraction (SPE) cartridges SAX**, 500 mg bed weight, 6 mL column volume, Supelco DSC-SAX (Supelco, St. Louis, MO) or Thermo HyperSep SAX (Thermo Scientific, Waltham, MA).
	15. **Disposable plastic syringe**, 10 mL (Becton Dickinson, Franklin Lakes, NJ), or equivalent.
	16. **Disposable plastic syringe,** 2 mL (Becton Dickinson), or equivalent.
	17. **Syringe-driven filter unit,** 0.22 µm, Millipore Millex GP (Bedford, MA), or equivalent.
	18. **HPLC amber vials,** 2 mL (Agilent Technologies), or equivalent.

## **PROCEDURE**

### Sample Preparation

#### Sample reconstitution Powder samples were reconstituted by dissolving 25 g powder sample and 50 mg α-amylase in 200 g warm water (40°C). The SRM was reconstituted by dissolving 10 g powder and 50 mg α-amylase in 90 g warm water (40°C). The samples were digested at 40°C for 15 min to let the enzyme work.

### Extraction

###  8.2.1 An aliquot of 15 g reconstituted sample or 15 g reconstituted RTF sample was weighed into a 100 mL amber glass volumetric flask.

### 8.2.2 40 mL extraction buffer (100 mmol/L phosphate buffer; 2% ascorbic acid; 0.1% DTT; pH 4.5) was added and the flask was then heated at 90°C for 30 min, while stirring.

### 8.2.3 After cooling to room temperature, 2 mL protease solution (4 mg/mL) was added and incubation was carried out in a water bath at 37°C for 30 min.

### 8.2.4 After cooling to room temperature, the volume was made up to the mark with water.

### 8.2.5 After filtration through folded paper filter, 10 mL filtratewas transferred to a 10 mL amber glass volumetric flask and 50 µL of 5 µg/mL IS solution was added.

### 8.2.6 From this solution, 3 mL was loaded on an SAX cartridge (previously conditioned with 4 mL acetonitrile and equilibrated with 10 mL extraction buffer).

### 8.2.7 After loading, the cartridge was washed with 6 mL extraction buffer and analytes were then eluted with 4 mL SPE eluting solution into amber glass tubes.

### 8.2.8 Eluate was then evaporated under controlled temperature at 55°C and nitrogen flow.

### 8.2.9 Extracts were then reconstituted in 1.5 mL reconstitution solution (H2O, 1% ascorbic acid, 0.5% DTT) and filtered through 0.22 µm membrane into an amber LC vial.

### UHPLC-MS/MS Analysis

### UHPLC conditions

5 µL of the reconstituted extract was injected onto an UHPLC system (Agilent 1290 Infinity) equipped with a Waters UHPLC HSS T3, 1.8 µm, 2.1 × 150 mm column. Mobile phase A consisted of H2O, 0.5% acetic acid. Mobile phase B was acetonitrile. Following injection, isocratic conditions of 0% of solvent B were initially used for 0.5 min, then a step direct to 10% of solvent B was achieved in 0.1 min. Isocratic conditions of 10% solvent B were held for 1.4 min and followed by a linear gradient to 25% solvent B for 3.5 min. Then, a step directly at 99% B was achieved in 0.1 min and held for 1.9 min before going back to start conditions (0% of solvent B) in 0.1 min. Start conditions were kept for 2.4 min.

#### Table1: Summary of gradient program

|  |  |  |  |
| --- | --- | --- | --- |
| TIME | FLOW (ML/Min) | % A | %B |
| 0.0 | 0.25 | 100 | 0.0 |
| 0.50 | 0.25 | 100 | 0.0 |
| 0.60 | 0.25 | 90 | 10 |
| 2.00 | 0.25 | 90 | 10 |
| 5.50 | 0.25 | 75 | 25 |
| 5.60 | 0.25 | 1.0 | 99 |
| 7.50 | 0.25 | 1.0 | 99 |
| 7.60 | 0.25 | 100 | 0.0 |
| 10.0 | 0.25 | 100 | 0.0 |

* + 1. **Mass spectrometer conditions** Mass spectrometry was performed on an Agilent 6460 MS in ESI+ mode operating at unit resolution. ESI capillary voltage was set at 3.5 kV; nozzle voltage, 600 V; gas temperature, 300°C; sheath gas temperature, 350°C; gas flow, 10 L/min; sheath gas flow, 12 L/min; nebulizer pressure 30 psi. Multiple-reaction monitoring mode was applied for quantification and compound identification confirmation. The transitions are shown in Table2. The dwell times were set up at 100, 200, and 75 msec for quantifier (Q), qualifier (q), and ISs, respectively.
		2. **Mass transitions** Mass transitions for each vitamin and its corresponding internal standard are given in Table 3. Retention time windows are also given in the table. Like the tune parameters, these parameters may need adjusted based upon instrument model.
		3. **System Suitability**

### RSD of retention time and peak area should not be higher than 5% for FA and 5-Me THF.

###  8.3.5 Specificity

### A tandem mass spectrometer was chosen as detection mode. Optimization consisted of selecting the precursor ion as well as the two main product ions for each analyte. The transition precursor ion/main product was defined as quantifier. The transition precursor ion/second main product ion was defined as qualifier. The ratio quantifier/qualifier was followed in all series with a defined limit to confirm peak identification.

**Table 2. MS/MS Transitions for Folic Acid and 5-Me THF**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Time Range****min** | **Analyte** | **Q1** | **Q3** | **Fragment or Voltage V** | **Collision energy,****eV** |
| 2.0-5.0 | 5- Me THF (Q) | 460.2 | 313.1 | 108 | 14 |
| 2.0-5.0 | 5- Me THF (q) | 460.2 | 180.0 | 108 | 42 |
| 2.0-5.0 | 5- Me THF IS | 465.2 | 313.1 | 120 | 15 |
| 5.0-8.0 | Folic Acid(Q) | 442.2 | 295.1 | 90 | 10 |
| 5.0-8.0 | Folic Acid(q) | 442.2 | 176.0 | 90 | 40 |
| 5.0-8.0 | Folic Acid IS | 447.1 | 295.0 | 92 | 10 |

Note that Collison energy and fragment voltage has to be tuned according to the make and model of mass spectrometer. The values pertain to Agilent 6460. This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by BIS of the product named. Equivalent products may be used if they can be shown to lead to the same results.

## **CALCULATIONS**

## To accurately calculate the final folic acid (FA) concentration (expressed in µg/mL) of the stock solution, consider the following: purity and water content.

##  Calculate final concentration as follows: Purity: x%, water content: y%, and weight: z mg.

## FA concentration = [z × 1000 × (x/100) × (1 – (y/100))]/50

## To express the final 5-Me THF concentration (expressed in µg/mL) of the stock solution in its free form, consider the following: purity, water content, molecular weight (MW) of the salt form, and MW of the free form.

## Calculate final concentration as follows: Purity: x%, water content: y%, weight: z mg, MW salt: 497.50 g/mol, and MW free form: 459.55 g/mol.

## 5-Me THF conc = [z × (459.55/497.50) × 1000 × (x/100) × (1 – (y/100))]/50

## Calculate the FA and 5-Me THF final content (= w1) separately, in mg/100 g of product, using the following equation:

##  For powder samples:

$$W1=C×\frac{\left( m1+m2\right)×V1×V3 ×100}{m1×m3×V2×1000}$$

where C = concentration in the test solution (ng/mL) of FA or 5-Me THF, calculated using the dedicated

calibration curve; m1=mass of the sample weight for slurry, in g (= 25 g); m2 = mass of water weight to

prepare the slurry, in g (= 200 g); m3 = mass of the test portion, in g (= 15 g); V1=volume of the of sample

extract, in mL (= 100 mL); V2=volume of sample loaded on SPE, in mL (= 3.0 mL); V3=volume of the

reconstituted sample, in mL (= 1.5 mL); 100 = conversion to 100 g basis; 1000 = conversion from ng to µg.

For liquid samples:

$$W1=C×\frac{ V1×V3×100}{m3×V2×1000}$$

where C = concentration in the test solution (ng/mL) of FA or 5-Me THF, calculated using the dedicated calibration curve; m3=mass of the test portion, in g (= 15 g); V1=volume of the of sample extract, in mL (= 100 mL); V2=volume of sample loaded on SPE, in mL (= 3.0 mL); V3=volume of the reconstituted sample, in Ml(= 1.5 mL); 100 = conversion to 100 g basis; 1000 = conversion from ng to µg. Verify that FA and 5-Me THF software calculated concentrations are >lower LOQ (LLOQ) and <upper LOQ (ULOQ).If calculated concentration is <LLOQ, then this concentration cannot be taken into account for vitamin B9 concentration. Folate (vitamin B9) concentration is the sum of folic acid plus 5-Me THF. Results in µg/100 g are expressed as folic acid in reconstituted product.

# AnnexA

(Informative)

Inter Laboratory Data

1. **BIBLIOGRAPHY**
2. AOAC 2013.13: Meisser-Redeuil, K., Bénet, S., Gimenez, C., Campos-Giménez, E., & Nelson, M. (2014). Determination of folate in infant formula and adult/pediatric nutritional formula by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry: First Action 2013.13. *Journal of AOACInternational*,*97*(4),121-1126.

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**Review of Indian Standards**

Amendments are issued to standards as the need arises on the basis of comments. Standards are also reviewed periodically; a standard along with amendments is reaffirmed when such review indicates that no changes are needed; if the review indicates that changes are needed, it is taken up for revision. Users of Indian Standards should ascertain that they are in possession of the latest amendments or edition by referring to the latest issue of ‘BIS Catalogue’ and ‘Standards: Monthly Additions’.

This Indian Standard has been developed from Doc No.: FAD 28 (17158).

### Amendments Issued Since Publication

**Amend No. Date of Issue Text Affected**