BUREAU OF INDIAN STANDARDS

DRAFT FOR COMMENTS ONLY

भारतीय मानक मसौदा

खाद्य पदार्थों में विटामिन बी9 (फोलेट्स) का निर्धारण अल्ट्रा-हाई-परफॉर्मेंस लिक्विड क्रोमैटोग्राफी - टेंडेम मास स्पेक्ट्रोमेट्री - परीक्षण की पद्धति

Draft Indian Standard

Determination of Vitamin B9 (Folates) in Foodstuffs by Ultra-High-Performance Liquid Chromatography - Tandem Mass Spectrometry – Method of Test

(*First Revision of IS 7234*)

ICS No 67.050

Test Methods for Food Products, Sectional Committee, FAD 28	Last date of comments 22/02/2025

FOREWORD

(Foreword shall be updated later)

Vitamin B9, or folate, is a water-soluble vitamin, 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 first published in 1974 with a title "*Method for estimation of folic acid in foodstuffs*" in which microbiological method was described for estimation of folic acid in food stuffs. The microbiological method is old and there is a lot of variability in this method so a need was felt to revise the standard. In the first revision of the standard, the microbiological method has been replaced by the method based on AOAC 2013.13 *"Determination of Folate in Infant Formula and Adult/Pediatric Nutritional Formula by Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry"*.

For the revision of the standard, a collaborative Research & Development (R&D) project with an objective to validate test methods of Vitamins in identified food matrices was assigned to CSIR - Central Food Technological Research Institute (CFTRI), Mysuru along with five participating laboratories. A multi-lab validation of the method described in AOAC 2013.13 has been performed by Nestle India Limited Laboratory Services (Moga), Eureka Analytical Services Pvt Ltd (Bengaluru), Eurofins Analytical Services India Pvt. Ltd. (Bengaluru), Microchem Silliker Pvt Ltd (Navi Mumbai) and CSIR-CFTRI on food matrices namely, cereals, fruit and vegetables. The performance characteristics obtained for these matrices have been referred in Annex A. The HORRATr values are within recommended value of less than 2.

In reporting the result of a test or analysis made in accordance with this standard, is to be rounded off, it shall be done in accordance with IS 2 : 2022 'Rules for rounding off numerical values (second revision)'.

1 SCOPE

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

2 REFERENCES

The standards given below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards:

IS No.	*Title*
IS 1070 : 2023	Reagent Grade Water Specification (*fourth revision*)

3 PRINCIPLE

Powder samples were reconstituted by dissolving 25 g powder sample and 50 mg α-amylase or Taka diastase 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 Liquid Chromatography (LC) vial for Ultra-High-Performance Liquid Chromatography - Tandem Mass Spectrometry (UHPLC-MS/MS) analysis.

4 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.

4.1 L-Ascorbic acid, CAS Registry Number® (CAS RN®): 50-81-7; Purity >=98%.

4.2 Ammonium acetate, CAS Registry Number® (CAS RN®): 631-61-8; ACS grade ; > 98 % purity.

4.3 1,4-Dithiothreitol, CAS Registry Number® (CAS RN®): 3483-12-3; For Biochemistry.

4.4 Disodium hydrogen phosphate powder, CAS Registry Number® (CAS RN®): 7558-79-4; purity >=99%.

4.5 Taka diastase from Aspergillus Oryzae, 100U/mg, CAS Registry Number® (CAS RN®): 9001-19-8.

4.6 α-Amylase from *Bacillus subtilis*, ~50 U/mg, CAS Registry Number® (CAS RN®): 9000-90-2.

4.7 Protease from *Streptomyces griseus*, Type XIV; >3.5 units/mg; CAS Registry Number® (CAS RN®): 9036-06-0.

4.8 Folic acid, CAS Registry Number® (CAS RN®): 59-30-3; purity ≥97% (on anhydrous basis), Certified Reference Material should be used.

4.9 (6R, S)-5-Me Tetrahydro folic acid calcium salt, CAS Registry Number® (CAS RN®): 151533-22-1; purity ≥95%; HPLC grade.

4.10 [$^{13}C_5$]-Folic acid

4.11 [$^{13}C_5$] -(6S)-5-Me Tetrahydro folic calcium salt

4.12 Formic acid LCMSMS grade, CAS Registry Number® (CAS RN®):64-18-6; purity ≥ 98%.

4.13 Acetic acid glacial, CAS Registry Number® (CAS RN®): 64-19-7, ≥99.5%, Glacial Extrapure.

4.14 Sodium hydroxide Pellets (low Chloride), CAS Registry Number® (CAS RN®): 1310-73-2 , ACS grade; purity ≥98%.

4.15 1 M Hydrochloric acid, CAS Registry Number® (CAS RN®): 7647-01-0.

4.16 Hydrochloric acid 37%, CAS Registry Number® (CAS RN®): 7647-01-0; ACS Reagent.

4.17 Ortho-phosphoric acid, 85% , CAS Registry Number® (CAS RN®): 7664-38-2; ACS reagent.

4.18 Ethanol, HPLC grade.

4.19 Methanol, HPLC grade.

4.20 Isopropanol, LC-MS grade.

4.21 Acetonitrile, LC-MS grade.

NOTE — CAS Registry Number® is a trademark of CAS corporation. 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.

5 APPARATUS

5.1 Column, C18, 1.8 µm; 2.1×150 mm

5.2 UHPLC-MS/MS system, UPLC coupled with triple quadrupole detector equipped with positive electrospray ionization (ESI) mode source operating at unit resolution, or equivalent.

5.3 Amber glassware, Standard laboratory Class A.

5.4 Micro pipet, Adjustable (volumes from 2 to 20 µL) and disposable tips.

5.5 Micro pipet, Adjustable (volumes from 10 to 100 µL) and disposable tips.

5.6 Micro pipet, Adjustable (volumes from 100 to 1000 µL) and disposable tips.

5.7 Analytical balance, Precision 0.1 mg.

5.8 Homogenizer

5.9 *p*H meter

5.10 Water bath (up to 90°C), with magnetic stirrers

5.11 Folded filters, 597½ grade.

5.12 Solid phase extraction (SPE) cartridges SAX, 500 mg bed weight; 6 mL column volume.

5.13 Disposable plastic syringe, 10 mL.

5.14 Disposable plastic syringe, 2 mL.

5.15 Syringe-driven filter unit, 0.22 µm.

5.16 HPLC amber vials, 2 mL.

6 PROCEDURE

6.1 Standard and Solution Preparation

6.1.1 *Mobile phases and prepared solutions*

6.1.1.1 *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.

6.1.1.2 *Mobile Phase B,* Acetonitrile.

6.1.1.3 *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.

6.1.1.4 *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.

6.1.1.5 *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.

6.1.1.6 *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.

6.1.1.7 *Dissolution solution A; Sodium hydroxide 0.1 mol/L 5% (v/v)–ethanol 20% (v/v)*

Into a 100 mL volumetric flask 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.

6.1.1.8 *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.

6.1.1.9 *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.

6.1.2 *Analytical standards*

6.1.2.1 *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 (**6.1.1.7**). Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

6.1.2.2 *5-Me THF stock standard (approximately 100 µg/mL)*

Into a 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 (**6.1.1.8**). Store in aliquots flushed with N2. This solution remains stable for 5months at –20°C.

6.1.2.3 *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 (**6.1.1.9**). Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

6.1.2.4 *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 (**6.1.2.3**). Make up to volume with dissolution solution C (**6.1.1.9**) Store in aliquots flushed with N2. This solution remains stable for 3 months at –20°C.

6.1.3 *Internal Standard Stock Solutions*

6.1.3.1 *[$^{13}C_5$]-Folic acid stock solution (approximately200 µg/mL)*

Into a 10 mL amber glass volumetric flask, weigh 2.00 ± 0.20 mg [$^{13}C_5$]-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 N_2. This solution remains stable for 5 months at –20°C.

6.1.3.2 *[$^{13}C_5$] -(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.

6.1.4 *Working Standard Solution Preparation*

6.1.4.1 *Internal Standard 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 (**6.1.1.9**). Store in aliquots flushed with N2. This solution remains stable for 5 months at –20°C.

6.1.4.2 *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 (**6.1.1.9**) 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.

6.2 Sample Preparation

Reconstitute the homogenized samples by dissolving 25 g powder sample and 50 mg α-amylase in 200 g warm water (40°C). Digest the samples at 40°C for 15 min to let the enzyme work. For cereals and sample containing high starch, increase the amount of α-amylase or taka diastase.

6.3 Extraction

6.3.1 An aliquot of 15 g reconstituted was weighed into a 100 mL amber glass volumetric flask.

6.3.2 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.

6.3.3 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.

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

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

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

6.3.7 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.

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

6.3.9 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.

6.4 UHPLC-MS/MS Analysis

6.4.1 *UHPLC conditions*

6.4.1.1 Inject 5 µL of the reconstituted extract onto an UHPLC system equipped with a C18 UPLC Column (1.8 µm, 2.1 × 150 mm).

6.4.1.2 The mobiles phases are as follows

a) Mobile phase A: Water and 0.5% acetic acid,
b) Mobile phase B: Acetonitrile.

6.4.1.3 Following injection, use isocratic conditions of 0% of solvent B initially for 0.5 min, then a step direct to 10% of solvent B will be achieved in 0.1 min.

6.4.1.4 Hold the isocratic conditions of 10% solvent B 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 should be achieved in 0.1 min and hold for 1.9 min before going back to start conditions (0% of solvent B) in 0.1 min. Keep the start conditions for 2.4 min. These values are indicative and need to be optimized for each instrument used. The gradient program for the column is given in Table 1.

Table 1 Summary of gradient program
(Clause 6.4.1.4)

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

6.4.2 *Mass spectrometer conditions*

6.4.2.1 Mass spectrometry to be performed in ESI+ mode operating at unit resolution. Set the ESI capillary voltage at 3.5 kV; nozzle voltage at 600 V; gas temperature as 300°C; sheath gas temperature as 350°C; gas flow as 10 L/min; sheath gas flow, 12 L/min; nebulizer pressure 30 psi. Apply the multiple-reaction monitoring mode for quantification and compound identification confirmation. The transitions are shown in Table 2. Set the dwell times at 100, 200, and 75 msec for quantifier (Q), qualifier (q), and ISs, respectively.

NOTE — The tune conditions and parameters described may be adjusted based upon instrument model.

6.4.2.2 *Mass transitions*

Mass transitions for each Vitamin and its corresponding internal standard are given in Table 2. Retention time windows are also given in the table. Like the tune parameters, these parameters may need to be adjusted based upon instrument model.

6.4.2.3 *System Suitability*

The Relative Standard Deviation (RSD) of retention time and peak area should not be higher than 5% for FA and 5-Me THF.

6.4.2.4 *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
(Clause 6.4.2.2)

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 — Collison energy and fragment voltage has to be tuned according to the make and model of mass spectrometer.

7 CALCULATIONS

7.1 Folic acid (FA) concentration (expressed in µg/mL)

To accurately calculate the final folic acid (FA) concentration (expressed in µg/mL) of the stock solution, consider the purity and water content. Calculate final concentration as follows using the Purity as x%, water content as y%, and weight as z mg.

$$\text{FA concentration} = [z \times 1000 \times (x/100) \times (1 – (y/100))]/50$$

7.2 5-Me THF concentration (expressed in µg/mL)

7.2.1 To express the final 5-Me THF concentration (expressed in µg/mL) of the stock solution in its free form, consider the purity, water content, molecular weight (MW) of the salt form, and MW of the free form. Calculate final concentration as follows using purity as x%, water content as y%, weight as z mg, Molecular Weight of salt equal to 497.50 g/mol, and Molecular Weight in free form equal to 459.55 g/mol.

$$\text{5-Me THF conc} = [z \times (459.55/497.50) \times 1000 \times (x/100) \times (1 – (y/100))]/50$$

NOTE — The molecular weights of the salts used should be checked.

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

the following equation:

For powder samples:

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

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:

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

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.

NOTE — (a) Verify that FA and 5-Me THF software calculated concentrations are > LOQ.
(b) If calculated concentration is <LOQ, then this concentration cannot be taken into account for Vitamin B9 concentration.

7.3 Reporting of Results

7.3.1 Folate (Vitamin B9) concentration is the sum of folic acid plus 5-Me THF.

7.3.2 Results in µg/100 g are expressed as folic acid.

Annex A
(*Foreword*)

PERFORMANCE CHARACTERISTICS OBTAINED DURING SINGLE LAB VALIDATION

Matrix	**Cereal (Wheat Flour)**	**Vegetables (Pea)**
Mean	15.712	119.620
SD	2.129	10.635
Reproducibility RSDR %	13.55	8.89
Predicted RSDR% (Horwitz)	10.57	7.79
HORRATR	1.28	1.14