BHUTAN STANDARD

METHODS FOR ESTIMATION OF FOLIC ACID IN FOODSTUFFS

UDC 664:543.867

BHUTAN STANDARD BUREAU
The National Standards Body of Bhutan
THIMPHU

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BTS 290: XXXX IS 7234: 1974

NATIONAL FOREWORD

This Bhutan Standard which is identical with IS 7234: 1974 METHODS FOR ESTIMATION OF FOLIC ACID IN FOODSTUFFS Standard issued by the Bureau of Indian Standards was adopted by Bhutan Standards Bureau by Food and Agriculture technical committee (TC 02) and approved by the Bhutan Standards Bureau Board (BSB Board) on xxxx, 2019.

The text of the IS Standard has been approved as suitable for publication as Bhutan Standard without deviation. Certain conventions are however, not identical to those used in Bhutan Standard.

Attention is particularly drawn to the following:

a) Where the words “IS Standard” appear referring to this standard, they should be read as “Bhutan Standard”.

b) Wherever page numbers are quoted, they are “IS Standard” page numbers.

Indian Standard

METHOD FOR ESTIMATION OF FOLIC ACID IN FOODSTUFFS

UDC 664 : 543·867 FOL

INDIAN STANDARDS INSTITUTION
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Indian Standard

METHOD FOR ESTIMATION OF FOLIC ACID IN FOODSTUFFS

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Indian Standard

METHOD FOR ESTIMATION OF FOLIC ACID IN FOODSTUFFS

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 21 January 1974, after the draft finalized by the Food Hygiene, Sampling and Analysis Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Vitamins are required to be assessed in a large number of foodstuffs, such as dairy products, animal feeds, processed cereals and other natural or manufactured foodstuffs. Moreover, different methods of vitamin assays are used in different laboratories. Therefore, with a view to establishing uniform procedures and also for facilitating a comparative study of results, ISI is bringing out a series of standards on vitamin assays. These would include chemical as well as microbiological methods, wherever applicable.

0.3 In the preparation of this standard, considerable assistance has been derived from National Institute of Nutrition, Hyderabad. Assistance has also been derived from the following publications:

CLEGG (K M), KODICEK (E) and MISTRY (S P). A modified medium for L. casei for assay of B vitamins. *Boichem J* 50, 1952; 326.

HERBERT (V), REBECCA (F) and KORTZ (B J). The assay and nature of folic acid activity in human serum. *J Clin Invest* 40, 1961; 81-91.

RAMASASTRY (B V) and LAXSHMIAH (N). Some studies on folyl or glutamyl carboxy peptidase from human plasma and its use in estimation of folic acid content of foods. *J Sci & indust Res* 29, Suppl 8, 1970; 551-54.

0.4 In reporting the result of an analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS : 2-1960*.

*Rules for rounding off numerical values (*revised*).

1. SCOPE

1.1 This standard specifies microbiological method for estimation of folic acid in foodstuffs.

2. QUALITY OF REAGENTS

2.1 Unless specified otherwise, pure chemicals and distilled water (*see* IS : 1070-1960*) shall be employed in the tests.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the results of analysis.

3. PREPARATION OF THE ASSAY SAMPLE

3.0 The technique used for preparing the material for the analysis is common to every vitamin determination. It should be ensured that the sample taken for the assay is representative of the whole, and any deterioration of the vitamin to be examined is prevented.

3.1 Powders and liquids should be mixed thoroughly until homogeneity is achieved. Dry materials, namely, bread, biscuits, and grains, should be ground and mixed thoroughly.

3.2 Wet or fresh material may be minced with a knife or scissors, or homogenized in a blender, if necessary, in the presence of the extracting solvent.

4. METHOD

4.0 Principle — The micro-organisms (*see* **4.2**) have specific requirement for folic acid for their growth. The growth response on a defined medium complete in all respects, except the folic acid under test, is proportional to the concentration of the folic acid added to the medium up to a certain range. Either the acid or the turbidity produced by the organisms is measured to determine the extent of growth and thereby the amount of folic acid in the test solution.

4.1 Forms of Folates in Foodstuffs — Foodstuffs usually contain the following forms of folates.

4.1.1 *Free Folates (Folic Acid or Monoglutamate)*

4.1.2 *Reduced Free Folates*

4.1.2.1 *Tetrahydro-folic acid (THFA)*

4.1.2.2 *10-formyl THFA*

*Specification for water, distilled quality (*revised*).

4.1.2.3 *5, 10-methenyl THFA*

4.1.2.4 *5, 10-methenyl THFA*

4.1.3 *5-Methyl THFA*

4.1.4 *Triglutamates*

4.1.5 *Heptaglutamates*

4.2 Test Organism

4.2.1 *Lactobacillus Casei* — ATCC No. 7469 or NCIM 2077 (For **4.1.1, 4.1.2. 4.1.3** and **4.1.4**).

4.2.2 For the estimation of heptaglutamate the food extract is incubated with folic acid conjugase and the monoglutamate formed is determined by *L. casei*.

NOTE — The organisms *Streptococcus faecalis* (ATCC No. 8043) and Pediococcus cerevisiae (ATCC No. 8081) may also be used in case of fractional estimation of different forms of folates.

4.3 Apparatus

4.3.1 *Incubator* — maintaining uniform temperature in the range 30 to 37°C. The temperature, however, should not vary by more than ± 0·5°C at a particular setting. A water-bath will also serve the purpose.

4.3.2 *Autoclave* — large enough to admit culture tubes in their racks and capable of accurate adjustment of pressure.

4.3.3 *Bacteriological Tubes* (*Rimless*) — size 15 mm × 150 mm or 25 mm × 200 mm.

4.3.4 *Cotton Plugs or Aluminium Caps* — to fit these tubes.

4.3.5 *Culture Tube Racks* — to hold vertically 120 tubes and so designed as to permit free circulation of air. Rust-proof metal racks are required.

4.3.6 *Inoculating Needle and Loop* — made of platinum or nichrome steel.

4.3.7 *Hypodermic Syringe* — 5- to 10-ml for inoculating the tubes.

4.3.8 *Refrigerator* — of sufficient capacity to hold reagents, media, stock solutions and the culture.

4.3.9 *Centrifuge* — electrically operated, laboratory model, to hold test-tubes.

4.3.10 *Sterilizing Oven* — for pipettes.

4.3.11 *Burette* — 25- or 50-ml, graduated to 0·1 ml.

4.3.12 *p*H *Meter or Lovibond Comparator* — with disc for adjustment of *p*H between 6 and 8.

4.3.13 *Conical Flasks* — 100- or 125-ml.

4.3.14 *Volumetric Flasks* — 100-ml and 2 litres.

4.3.15 *Graduated Pipettes* — 5- or 10-ml.

4.3.16 *Volumetric Pipettes* — 5-, 10- and 20-ml.

4.3.17 *Photoelectric Colorimeter or Turbidimeter*

4.3.18 *Other Apparatus* — like funnels, flasks, glass-stoppered cylinders and bottles as necessary. Automatic dispenser titrator, if available, will be useful.

4.4 Reagents

4.4.1 *Casein Hydrolysate*

4.4.1.1 Stir 100 g of vitamin-free casein or casein hydrolysate (acid digested) (*see* IS : 7203-1973*) with 250 ml of 95 percent ethyl alcohol for 15 minutes in an 800-ml beaker and filter with suction. Repeat using another 250-ml of alcohol. Transfer the alcohol-washed casein into a round-bottom flask of at least 1-litre capacity, preferably one having two necks ground to standard taper. Mix well with 500 ml of constant boiling hydrochloric acid. Fit the flask with a glass stopper and a water-cooled condenser and reflux over a low flame or hot plate for 8 to 12 hours. Use 1 : 1 HCl solution for the hydrolysis. Heat carefully and gradually to avoid frothing during initial stages of hydrolysis. Mix the contents of the flask by shaking it occasionally. Keep a wet towel ready to cool the flask if the reaction becomes too vigorous.

4.4.1.2 After refluxing, fit the flask with a condenser and receiving flask suitable for vacuum distillation and remove as much HCl as possible by concentrating the hydrolysate to a thick paste under reduced pressure. Introduce air through a bleeder tube well into the bottom of the flask to minimize bumping during the final stages of the concentration. The temperature at which the distillation is carried out should be 70 to 80°C. To get rapid and complete distillation at this low temperature, reduce the pressure considerably by a steam aspirator or a vacuum pump. Take care to trap HCl fumes effectively, especially with a vacuum pump.

4.4.1.3 Re-dissolve the paste in approximately 200 ml of water and repeat the concentration to remove additional amounts of HCl; if a satisfactory hydrolysate has not been attained with a single concentration. Dissolve the hydrolysate paste in about 700 ml of water and adjust the *p*H to 3·5 with 40 percent NaOH. Decolourize by stirring with 20 g of activated charcoal at room temperature to remove residual niacin and folic acid. Stir until a small test filtrate gets light straw coloured. This step removes any niacin which might have remained in the alcohol-washed casein. Filter through a large fluted filter or by suction as preferred.

*Specification for casein hydrolysate (acid digested) microbiological grade.

4.4.1.4 Adjust the *p*H of the filtrate to 6·8, dilute to 1 litre, and store under toluene and over chloroform in the refrigerator. Occasionally, a precipitate will form in this solution on standing. This is mainly tyrosine. It is a good practice to shake up the solution and use the suspended material as well as fluid portion. The insoluble material will dissolve when the entire medium is prepared.

4.4.2 *Activated Charcoal* — Weigh about 100 g of animal charcoal. Add 250 ml of hydrochloric acid (1 : 1) and boil for 2 to 3 hours. Dilute the mixture with hot distilled water. Filter with a Buckner funnel and water pump and wash repeatedly with boiling distilled water until the filtrate is no longer acidic. Drain off the water and dry in an oven at 110°C for 1 hour.

4.4.3 *l-cystine* — Suspend 4·0 g of cystine in water using concentrated hydrochloric to aid solution and make up to 500 ml with water.

4.4.4 *dl-tryptophane* — Dissolve 2·0 g of tryptophane in water using concentrated hydrochloric acid to aid solution. Adjust the *p*H to 3·5 and make up the volume to 500 ml. Stir with activated charcoal (2 g per 100 ml) for 10 to 15 minutes, filter and store in the cold under toluene.

4.4.5 *Adenine-Guanine-Uracil (AGU) Solution* — Dissolve adenine sulphate, guanine hydrochloride and uracil (0·2 g each) in water using concentrated hydrochloric acid to aid solution, and make up the volume to 100 ml.

4.4.6 *Xanthine* — Dissolve 0·2 g of xanthine in water using ammonia to aid solution and make up to 100 ml with water.

4.4.7 *Salt A* — Dissolve 25·0 g each of dibasic potassium phosphate (K_2HPO_4) and monobasic potassium phosphate (KH_2PO_4) in 250 ml of water.

4.4.8 *Salt B* — Dissolve the following salts in water to make 250 ml of concentrated solution. Add a few drops of concentrated hydrochloric acid to obtain a clear solution:

Magnesium sulphate ($MgSO_47H_2O$)	10·0 g
Sodium chloride (NaCl)	0·5 g
Ferrous sulphate ($FeSO_47H_2O$)	0·5 g
Manganese sulphate ($MnSO_44H_2O$)	0·5 g

4.4.9 *Pyridoxine and Thiomine* — Dissolve 50 mg of pyridoxine or equivalent amount of pyridoxine hydrochloride and 200 mg of thiamine or equivalent amount of thiamine hydrochloride in water and make up to 100 ml.

4.4.10 *Biotin* — Take 250 mg of biotin in 500 ml of 50 percent alcohol in water.

4.4.11 *Riboflavin* — Make up 20·0 mg of riboflavin with water to 100 ml, using acetic acid to help solution. (If necessary the solution may have to be heated on a water bath.)

4.4.12 *Nicotinic Acid* — 50 mg in 500 ml of 50 percent alcohol in water.

4.4.13 *p-Aminobenzoic Acid* (*PABA*) — 12·5 mg in 100 ml water.

4.4.14 *Calcium Pantothenate* — 54·4 mg in 500 ml water.

4.4.15 *Peptone Solution* — Dissolve 10 g of peptone (*see* IS : 6853-1973*) in 80 ml water, adjust the *p*H to 3·0 with HCl and make up the volume. Stir the solution with 5 g of activated charcoal (*see* **4.4.2**) for one hour and filter. Repeat this process twice more with 2 g of charcoal.

4.4.16 *dl Alanine* — 2 g in 100 ml water.

4.4.17 *Phosphate-Buffers* — *p*H 7·2. Dissolve 27·23 g of monobasic potassium phosphate (KH_2PO_4) and 5·60 g of sodium hydroxide in water and dilute to 1 litre.

4.4.18 *Ascorbic Acid, 1 percent — Charcoal Treated* — Dissolve 1 g of a ascorbic acid in 20 ml water. Add to the solution about 500 to 600 mg of activated charcoal (*see* **4.4.2**) stir the mixture gently for 15 minutes, filter and add an equal volume of phosphate buffer. Adjust the *p*H of the solution to **6.1** and further dilute with an equal volume of water. This solution should be prepared fresh on the day of assay.

4.4.19 *Folic Acid Standard Solution*

4.4.19.1 *Stock standard* — Dissolve 10·0 mg of folic acid in 100 ml of 0·8 percent sodium bicarbonate solution to obtain 100 µg/ml. Stock solution may also be prepared in 0·01 sodium hydroxide in 20 percent ethanol. The solution should be covered with toluene and stored in coloured bottle in refrigerator.

4.4.19.2 *Working standard* — Dilute the stock solution to get a final concentration of 0·1 m µg/ml for assays with *L. casei*.

4.4.19.3 *Standard range* — L. casei : 0 to 0·4 m µg/ml.

4.4.20 *Composition of Basal Medium for L. Casei* — for 100 ml :

Casein hydrolyzate	10 ml
Peptone	0·4 ml
dl-tryptophan	10 ml
l-cystine	5 ml
dl-alanine	1 ml

*Specification of peptone, microbiological grade.

Salt A	5 ml
Salt B	1 ml
AGU solution	1 ml
Xanthine	1 ml
Biotin (1·5 ml of stock diluted to 100 ml)	1 ml
Thiamine-pyridoxine solution	1 ml
Riboflavin	1 ml
Nicotinic acid	2 ml
Ca-pantothenate	2 ml
PABA	0·3 ml
Glucose	4 g
Sodium acetate. $3H_2O$	6·64 g

Adjust the *p*H to 6·8 and make up the volume to 100 ml and filter the solution.

4.4.21 *Sodium Phosphate Buffer* 0·2 *M* — *p*H 6·1.

4.4.21.1 *Sodium phosphate, monobasic* (NaH_2PO_4) — 31·2 g/l.

4.4.21.2 *Sodium phosphate, dibasic* (Na_2HPO_4) — 28·4 g/l-mix 80 ml of **4.4.21.1** and 15 ml of **4.4.21.2** with 100 ml of water.

5. PREPARATION OF SAMPLE FOR THE ASSAY

5.1 Take 1·0 g dry material or 10·0 g fresh homogenized material, add 40 ml of 0·1 m phosphate buffer, *p*H 7·2, and 2·5 ml, 4 percent ascorbic acid solution (after treating with charcoal). Autoclave the mixture for 15 minutes at 123°C and cool to room temperature. Adjust the *p*H to 4·5, make up the volume to 100 ml and filter. The filtrate is used for free folate estimation after suitable dilution. For the estimation of total folate, incubate 5 ml of the filtrate, 4 ml of 0·2 m acetate buffer *p*H 4·5, 1 ml of 100 m mercapto-ethanol and 0·2 ml of human plasma or chicken pancreas or hog kidney (*see* Appendix A) and few drops of toluene over night at 37°C. After incubation, inactivate the enzyme by heating in a boiling water bath for 3 minutes. Use aliquots of the filtrate for the assay. A final concentration of 0·1 m µg/ml for *L. casei*.

5.1.1 Take 0·5, 1·0, 2·0 and 3·0 ml in duplicate. Add 1·0 ml of ascorbic acid (0·25 percent) and sufficient water to bring the volume to 5·0 ml followed by 5·0 ml of the basal medium.

5.1.2 *Standard Levels* — Take 0, 0·5, 1·0, 2·0, 3·0 and 4·0 ml of the working standard solution in duplicate and add 1·0 ml of 0·25 percent ascorbic acid and sufficient water to bring the level to 5·0 ml followed by the addition of 5·0 ml of basal medium.

6. PROCEDURE

6.1 Preparation of the Standard Tubes

6.1.1 *L. Casei* — Conc-0·1 m µg/ml dispense 0 to 4·0 ml of the working standard in duplicate add 1·0 ml of 0·25 percent ascorbic acid. Add sufficient water to bring the level to 5·0 ml followed by addition of 5·0 ml of basal medium. Cover the tubes with cotton and brown paper, autoclave at 120 to 123°C for 12 minutes and cool to room temperature.

6.2 Preparation of Assay Tubes — Dispense 0 to 0·3 ml of the test material in duplicate and add 1·0 ml of 0·25 percent ascorbic acid and sufficient water to bring the volume to 5·0 ml followed by 5·0 ml basal medium. Cover the tubes with cotton and brown paper, autoclave at 120 to 123°C for 12 minutes and cool to room temperature.

6.3 Inoculation and Incubation — Aseptically inoculate each tube with one drop of inoculum except the blank tube. Incubate at 37°C for approximately 18 hours.

6.4 Turbidimetric Method — At the end of the incubation period, remove the assay tubes and steam for 5 minutes. Cool and read turbidimetrically in a colorimeter at 660 nm using the uninoculated blank tube to set the instrument to zero.

6.5 Calculation — A standard curve for the assay is drawn by plotting the optical density or turbidity reading on the X-axis against concentration of the vitamin on the Y-axis. The vitamin content of the tubes in the unknown series is determined by interpolation of the colorimeter readings on the standard curve. The average for one ml of test solution is calculated from values obtained from not less than three tubes which do not vary by more than 10 percent on the average. The vitamin content of the test solution is calculated using the following relationship:

$$\mu g \text{ of folic acid/g sample} = \frac{\text{Average } \mu g/ml \times \text{diluting factor}}{\text{mass of the sample}}$$

6.6 Titrimetric Method

6.6.1 Transfer the contents of each tube to a 125 ml conical flask and rinse the tube once with about 10 ml water, adding the rinsing to the flask. Add about 0·2 ml of 0·1 percent bromothymol blue and titrate with 0·1 N NaOH to a green colour, about 6·8 *p*H. Hold a flask for reference colour for about 10 to 20 titrations and then substitute a new flask.

6.6.2 *Calculation* — Draw a standard curve for the assay by plotting ml of 0·1 N NaOH used in titration of the standard tubes against mmcg of folic acid per tube in the standard series. Determine the folic acid content of

the tubes in the unknown series by interpolations of the titre values on the standard curve. Discard any values which show more than 1·00 mmcg or less than 0·1 mmcg folic acid per tube. Calculate the folic acid content for each of the duplicate sets of tubes. Calculate the folic acid content of the test material from the average of the values for each ml of test solution obtained from not less than 3 sets of these tubes which do not vary by more than 10 percent from the average, using the following formula:

$$\text{mmcg per g of sample} = \frac{\text{Average mmcg/ml} \times \text{volume}}{\text{mass of sample}} \times \text{dilution factor}$$

APPENDIX A

(*Clause* 5.1)

PREPARATION OF CONJUGASE

A-1. CHICKEN PANCREAS

A-1.1 Prepare by grinding the pancreas with five times its mass of distilled cold acetone. Leave the mixture at the ambient temperature overnight. Filter the precipitate. Wash with acetone and air dry powder and store the dry material in the refrigerator. To aliquot of the filtrate (5 or 10 ml), add 20 mg of chicken pancreas powder, make it to a paste with one drop of glycerine and incubate overnight with a few drops of toluene. Run as an enzyme blank with similar incubation mixture using the buffer only and the same amount of chicken pancreas.

A-2. HOG KIDNEY

A-2.1 Take fresh hog kidney in waring blender and blend with 3 ml of water per gram of kidney. Centrifuge the suspension and filter the supernatant solution. Freeze in 10-ml portions until ready for use.

A-2.2 Sodium Acetate Buffer (1 Percent) — Dissolve 5·0 g of anhydrous sodium acetate or 8 g of hydrated sodium acetate in 400 ml water. Adjust *p*H to 4·5 with acetic acid and make volume up to 500 ml.

INDIAN STANDARDS

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FOOD HYGIENE, SAMPLING AND ANALYSIS

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5399-1969	Methods for estimation of riboflavin (vitamin B_2) in foodstuffs
5400-1969	Methods for estimation of nicotinic acid (niacin) in foodstuffs
5401-1969	Methods for detection and estimation of coliform bacteria in foodstuffs
5402-1969	Method for plate count of bacteria in foodstuffs
5403-1969	Methods for yeast and mould count of foodstuffs
5404-1969	Code of practice for handling of food samples for microbiological analysis
5835-1970	Method for estimation of vitamin D in foodstuffs
5837-1970	Code for hygienic conditions for soft drinks manufacturing units
5838-1970	Method for estimation of vitamin C in foodstuffs
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6851-1973	Meat extract, microbiological grade
6852-1973	Bile salts, microbiological grade
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6854-1973	Methods of sampling and test for ingredients used in media for microbiological work
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7005-1973	Code for hygienic conditions for production, processing, transportation and distribution of milk
7127-1973	Tryptone, microbiological grade
7128-1973	Proteose peptone, microbiological grade
7203-1973	Casein hydrolysate (acid digested), microbiological grade
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7234-1974	Method for estimation of folic acid in foodstuffs
7235-1974	Method for estimation of tocopherols (vitamin E)

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117/418 B Sarvodaya Nagar, KANPUR 208005	82 72
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