BHUTAN STANDARD

METHODS FOR ESTIMATION OF VITAMIN B_{12} IN FOODSTUFFS

UDC 664:543.867

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BTS 289: XXXX IS 7529: 1975

NATIONAL FOREWORD

This Bhutan Standard which is identical with IS 7529: 1975 METHODS FOR ESTIMATION OF VITAMIN B12 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.

IS : 7529 - 1975

Indian Standard

(Reaffirmed 2005)
(Reaffirmed 2015)

METHOD FOR ESTIMATION OF VITAMIN B_{12} IN FOODSTUFFS

UDC 664 : 543·867 B_{12}

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METHOD FOR ESTIMATION OF VITAMIN B_{12} IN FOODSTUFFS

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Indian Standard

METHOD FOR ESTIMATION OF VITAMIN B_{12} IN FOODSTUFFS

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 16 January 1975, 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 processed cereals, dairy products, animal feeds, 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 and as well as microbiological methods, where applicable.

0.3 In the formulation of this standard, considerable assistance has been derived from Pharmacopoeia of the United States of America, XVII 1965.

0.4 In reporting the result of a test or 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*.

1. SCOPE

1.1 This standard prescribes a microbiological method for the estimation of vitamin B_{12} in foodstuffs.

2. PRINCIPLE

2.1 The micro-organism (*Lactobacillus leichmannii*) has specific requirement for vitamin B_{12} for its growth. The growth response on a defined medium complete in all respect, except the vitamin B_{12} under test, is proportional to the concentration of the vitamin B_{12} added to the medium up to a certain range. The turbidity produced by the organism is measured to determine the extent of growth and thereby the amount of vitamin B_{12}.

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

3. REAGENTS AND MEDIA

3.1 Reagents

3.1.1 *Quality of Reagents* — Unless specified otherwise, pure chemicals and distilled water (*see* IS : 1070-1960*) shall be employed in tests.

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

3.1.2 *Casein Hydrolysate*

3.1.2.1 Stir 100 g of ' vitamin-free ' casein hydrolysate (*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 one-litre capacity, and preferably having two necks ground to standard taper. Mix well with 500 ml of constant boiling hydrochloric acid (HCl). Fit the flask with a glass stopper and water-cooled condenser and reflux over a low flame or hot plate for 8 to 12 hours. Use HCl solution (1 : 1) for the hydrolysis. Heat gradually to avoid frothing during the initial stages of hydrolysis. Mix the contents of the flask by shaking and keep a wet towel ready to cool the flask if the reaction becomes too vigorous.

3.1.2.2 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 placed 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 not exceed that of a boiling water bath. Temperatures of 70 to 80°C, have been recommended. To get rapid and complete distillation at this low temperature it is necessary to reduce the pressure considerably. This may not be possible with a water aspirator unless high water pressure is available. A steam aspirator or a vacuum pump can be used. Take care to trap HCl fumes effectively, especially with a vacuum pump.

3.1.2.3 Re-dissolve the paste in approximately 200 ml of water and repeat the concentration to remove additional amounts of HCl, if necessary. The acid concentration should be sufficiently low so that subsequent neutralization will not yield salt, which might retard bacterial growth on the basal medium.

3.1.2.4 Dissolve the hydrolysate paste in about 700 ml of water and adjust the *p*H to 3·5 with 40 percent sodium hydroxide. Decolourize by stirring with 20 g of activated charcoal at room temperature. Stir until

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

†Specification for casein hydrolysate (acid digested), microbiological grade.

a small test filtrate is light straw colour. The decolourization may be complete in 5 minutes or may require more than an hour depending upon the charcoal used. This step removes any niacin and residual vitamin B_{12} which may have remained in the alcohol-washed casein. Filter through a large fluted filter or by suction.

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

3.1.3 *Sodium Acetate Buffer* (0·1 *M*) — Dissolve 6·8 g of sodium acetate and 2·9 ml of glacial acetic acid in distilled water and dilute to 500 ml.

3.1.4 *Asparagine Solution* — Dissolve 2·0 g of l-asparagine in water to make 200 ml. Store under toluene in a refrigerator.

3.1.5 *Adenine-Guanine-Uracil* (*AGU*) *Solution* — Dissolve 200 mg each of adenine sulphate, guanine hydrochloride, and uracil, by heating in 10 ml of dilute hydrochloric acid (1 : 3), cool, and add water to make 200 ml. Store under toluene in a refrigerator.

3.1.6 *Xanthine Solution* — Suspend 0·2 g of xanthine in 30 to 40 ml of water, heat to about 70°C, add 6·0 ml of ammonia, and stir until the solid is dissolved. Cool, and add water to make 200 ml. Store under toluene in a refrigerator.

3.1.7 *Salt Solution A* — Dissolve 10 g of monobasic potassium phosphate (K_2HPO_4) and 10 g of dibasic potassium phosphate (KH_2PO_4) in water to make 200 ml. Add 2 drops of hydrochloric acid, and store under toluene.

3.1.8 *Salt Solution B* — Dissolve 4·0 g of magnesium sulphate, 0·2 g of sodium chloride, 0·2 g of ferrous sulphate and 0·2 g of manganese sulphate in water to make 200 ml. Add 2 drops of hydrochloric acid, and store under toluene.

3.1.9 *Polysorbate 80 Solution* — Dissolve 20 g of polysorbate 80 in sufficient quantity of alcohol to make 200 ml. Store in a refrigerator.

3.1.10 *Vitamin Solution I* — Dissolve 10 mg of riboflavin, 10 mg of thiamine hydrochloride, 0·1 mg of biotin, and 20 mg of nicotinic acid in sufficient quantity of 0·02 N acetic acid to make 400 ml. Store, protected from light, under toluene in a refrigerator.

3.1.11 *Vitamin Solution II* — Dissolve 20 mg of para-aminobenzoic acid, 10 mg of calcium pantothenate, 40 mg of pyridoxine hydrochloride, 40 mg of pyridoxal hydrochloride, 8 mg of pyridoxamine dihydrochloride, and 2 mg of folic acid in sufficient quantity neutralized alcohol (1 : 4) to make 400 ml. Store, protected from light, in a refrigerator.

3.1.12 *Tomato Juice Preparation* — Centrifuge commercially canned tomato juice so that most of the pulp is removed. Suspend about 5 g per litre of analytical filter-aid in the supernatant liquid and filter, with the aid of reduced pressure, through a layer of filter-aid. Repeat, if necessary, until a clear, straw-coloured filtrate is obtained. Store under toluene in a refrigerator.

3.1.13 *Standard Cyanocobalamin Stock Solution* — To a suitable quantity of cyanocobalamin reference standard conforming to Indian Pharmacopoeia or equivalent, accurately weighed, add sufficient quantity of 25 percent ethyl alcohol to make a solution containing in each ml 1·0 μg of cyanocobalamin. Store in a refrigerator.

3.1.14 *Standard Cyanocobalamin Solution* — Dilute a suitable volume of standard cyanocobalamin stock solution with distilled water to a measured volume such that after the incubation period as described in **6**, the difference in transmittance between the inoculated blank and the 5·0 ml level of the standard cyanocobalamin solution is not less than that which corresponds to a difference of 1·25 mg in dried-cell mass. This concentration usually falls between 0·01 and 0·04 millimicrogram per ml of standard cyanocobalamin solution. Prepare a fresh standard solution for each assay.

3.2 Media

3.2.1 *Basal Medium, Stock Solution* — Add the ingredients in the order listed, carefully dissolving the cystine and tryptophan in the hydrochloric acid before adding the next eight solutions in the resulting solution. Add 100 ml of water, mix, and dissolve the dextrose, sodium acetate and ascorbic acid. Filter, if necessary, add the polysorbate 80 solution, adjust to *p*H 6·0 with 1N sodium hydroxide and add purified water to make 250 ml.

l-Cystine	0·1 g
l-Typtophan	0·05 g
Hydrochloric acid (IN)	10 ml
Adenine-Guanine-Uracil Solution (*see* **3.1.5**)	5 ml
Xanthine Solution (*see* **3.1.6**)	5 ml
Vitamin Solution I (*see* **3.1.10**)	10 ml
Vitamin Solution II (*see* **3.1.11**)	10 ml
Salt Solution A (*see* **3.1.7**)	5 ml
Salt Solution B (*see* **3.1.8**)	5 ml
Asparagine Solution (*see* **3.1.4**)	5 ml
Casein Hydrolysate Solution (*see* **3.1.2**)	25 ml
Dextrose Anhydrous	10 g
Sodium Acetate, Anhydrous (*see* **3.1.3**)	5 g
Ascorbic Acid	1 g
Polysorbate 80 solution (*see* **3.1.9**)	5 ml

3.2.2 *Culture Medium* — Dissolve 0·75 g yeast extract (*see* IS : 7004-1973*), 0·75 g of peptone (*see* IS : 6853-1973†), 1 g of anhydrous dextrose, and 0·2 g of potassium biphosphate in 60 to 70 ml of water. Add 10 ml of tomato juice (*see* **3.1.12**) and 1 ml of polysorbate 80 solution (*see* **3.1.9**). Adjust the *p*H of solution to 6·8 with sodium hydroxide, and add water to make 100 ml. Place 10 ml portions of the solution in test tubes, and plug with cotton. Sterilize the tubes and contents in an autoclave at 121°C for 15 minutes. Cool as rapidly as possible to avoid colour formation resulting from over-heating of the medium.

3.2.3 *Suspension Medium* — Dilute a measured volume of basal medium stock solution (*see* **3.2.1**) with an equal volume of water. Place 10-ml portions of the diluted medium in test tubes. Sterilize and cool as directed above for the culture medium.

4. PREPARATION OF SAMPLE

4.0 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. Powders and liquids should be mixed thoroughly until homogeneity is achieved. Dry materials like bread, biscuits, grains, etc, should be ground and mixed thoroughly. 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.

4.1 Extraction of Vitamin B_{12} From Crude Materials — It is desirable to convert the more labile forms of vitamin B_{12} to cyanocobalamin by cyanide treatment, and hence it has been common to extract B_{12} from crude materials with potassium cyanide (KCN) solution. The effective treatment with cyanide to be extracted with 0·01 percent potassium cyanide (KCN) (10 mg of KCN for 10 μg of vitamin B_{12} activity) *p*H 4·5 to 5·0 at 60°C for 30 minutes. Higher temperatures are necessary to release bound forms of vitamin B_{12}. Vitamin B_{12} has a maximum of stability at *p*H 4 to 5; and acetate buffer *p*H 4·6 is recommended when higher temperatures are used.

4.1.1 The absence of a response to vitamin B_{12} may be due not only to bound forms, but also to the presence of inhibitors.

4.2 Assay Preparation — Place a suitable quantity of the material to be assayed, accurately measured or weighed, in an appropriate vessel containing for each g or ml of sample taken, 25 ml of an aqueous extracting solution prepared just prior to use. It should contain, in each 100 ml, 1·29 g of disodium phosphate, 1·1 g of anhydrous citric acid, and 1·0 g of sodium metabisulphite. Autoclave the mixture at 121°C for 10 minutes. Allow any undissolved particles of the sample extract to settle, and filter or centrifuge, if necessary.

*Specification for yeast extract, microbiological grade.

‡Specification for peptone, microbiological grade.

Dilute an aliquot of the clear solution with water so that the final test solution contains vitamin B_{12} activity approximately equivalent to that of the standard cyanocobalamin solution added to the assay tubes.

5. TEST ORGANISM

5.1 Stock Culture of the *Lactobacillus leichmannii* — To 100 ml of culture medium, add 1·0 to 1·5 g of agar (*see* IS : 6850-1973*), and heat the mixture, with stirring on a steam bath, until the agar dissolves. Place approximately 10-ml portions of the hot solution in test tubes, cover the tubes suitably, sterilize at 121°C for 15 minutes in an autoclave (exhaust line temperature), and allow the tubes to cool in an upright position. Inoculate three or more of the tubes, by stab transfer of a pure culture of *Lactobacillus leichmannii*, ATCC No. 7830. Before first using a fresh culture in this assay, make at least ten successive transfers of the culture in two-week period. Incubate 16 to 24 hours at any selected temperature between 30 and 40°C but held constant to within ± 0·5°C, and finally store in a refrigerator.

5.1.1 Prepare fresh stab cultures at least three times each week, and do not use them for preparing the inoculum, if more than 4 days old. The activity of the micro-organism can be increased by daily or twice-daily transfer of the stab-culture, to the point where definite turbidity in the liquid inoculum can be observed 2 to 4 hours after inoculation. A slow-growing culture seldom gives a suitable response curve, and may lead to erratic results.

5.2 Inoculum — Make a transfer of cells from the stock culture of *Lactobacillus leichmannii* to a sterile tube containing 10 ml of the culture medium. Incubate this culture for 6 to 24 hours at any selected temperature between 30 and 40°C but held constant to within ± 0·5°C. Centrifuge the culture and decent the supernatant liquid under aseptic conditions. Suspend the cells from the culture in 10 ml of sterile suspension medium. Dilute an aliquot with sterile suspension medium to give transmittance that corresponds to a cell mass (as given in **5**) of 0·5 mg to 0·75 mg per tube when read against the suspension medium set at 100 percent transmittance.

6. PROCEDURE

6.1 Cleanse by suitable means, followed preferably by heating at 250°C for 2 hours, hard-glass test tubes, about 20 × 150 mm in size, and other glassware as the test organism is highly sensitive to minute amounts of vitamin B_{12} activity and to traces of many cleansing agents.

6.1.1 Add in triplicate, 1·0 ml or 1·5 ml, 2·0 ml, 3·0 ml, 4·0 and 5·0 ml respectively, of the Standard Cyanocobalamin Solution to the test tubes. To each tube and to four similar tubes containing no standard cyanocobalamin solution, add 5·0 ml of basal medium stock solution and sufficient water to make 10 ml.

*Specification of agar, microbiological grade.

6.1.2 To similar test tubes, add in triplicate, 1·0 ml or 1·5 ml, 2·0 ml, 2·0 ml and 4·0 ml of the assay preparation respectively. To each tube, add 5·0 ml of basal medium stock solution and sufficient distilled water to make 10 ml. Place one complete set of standard and assay tubes together in one tube rack and the triplicate set in a second rack or section of a rack, preferably in random order.

Cover the tubes suitably to prevent bacterial contamination, and sterilize the tubes and contents in an autoclave at 121°C for 15 minutes, arranging to reach this temperature in not more than 10 minutes by preheating the autoclave, if necessary. Cool as rapidly as practicable to avoid colour formation resulting from overheating of the medium. Take precautions to maintain uniformity of sterilizing and cooling conditions throughout the assay, since packing tubes too closely in the autoclave, or overloading it, may cause variation in the heating rate.

6.1.3 Aseptically add one drop of inoculum to each tube so prepared except to two of the four containing no standard cyanocobalamin solution (the uninoculated blanks). Incubate the tubes at a temperature between 30 and 40°C held constant to within ±0·5°C, until, following 16 to 24 hours of incubation, there has been no substantial increase in turbidity in the tubes containing the highest level of standard during 2-hour period.

Determine the transmittance of the tubes by mixing the contents of each tube, to which one drop of a suitable antifoam agent solution may be added, and transfer to an optical container. After agitating its contents, place the container in a spectrophotometer that has been set at a specific wavelength between 540 nm and 660 nm, and read the transmittance when a steady state is reached. This steady state is observed a few seconds after agitation when the reading remains constant for 30 seconds or more. Allow approximately the same time interval for the reading on each tube.

6.1.4 With the transmittance set at 1·00 for the uninoculated blank, read the transmittance of the inoculated blank. If this transmittance reading corresponds to a dried-cell mass greater than 0·6 mg per tube, or if there is evidence of contamination with a foreign micro-organism, disregard the results of the assay.

Then with the transmittance set at 1·00 for the inoculated blank, read the transmittance for each of the remaining tubes. Disregard the results of the assay if the difference between the transmittance observed at the highest level of the standard and that of the inoculated blank is less than the difference corresponding to a dried-cell mass of 1·25 mg per tube.

6.2 Identification of Vitamin B_{12}

6.2.1 Determine the absorbancy of the solution prepared for the assay in a 1 cm of quartz cell with a suitable spectrophotometer, using water as the

blank. Maxima within ±1 nm are found at 278 and 361 nm and within ±4 nm at 548 nm. The ratios of absorbancies are as follows:

$\frac{d_{361}}{d_{278}}$ is not less than 1·62 and not more than 1·88

$\frac{d_{361}}{d_{548}}$ is not less than 2·83 and not more than 3·45

6.2.2 *Assay* — Weigh out accurately on a microbalance, about 2 mg of vitamin B_{12}, transfer to a 50-ml volumetric flask with the aid of 15 or 20 ml water. Add water to make exactly 50 ml, and mix well. Determine the absorbency of the solution in a 1-cm quartz cell, at 361 nm with a suitable spectrophotometer, using water as blank. Calculate the percent purity of vitamin B_{12} by the following formula:

$$\frac{d_{361}}{0{\cdot}020\,7} \times \frac{1}{\text{sample mass (mg/10 ml)}} \times \frac{100}{100 - \text{loss on drying}}$$

The final stock solution for microbiological assays is prepared to contain, for example 10 μg of pure vitamin B_{12} and 10 mg of KCN per ml in 0·1 M acetate buffer pH = 4·6. The solution is dispensed in 1 ml ampoules which are sealed, sterilized at 100°C for 20 minutes and stored in a refrigerator for not more than one year. A new ampoule should be used for each assay.

7. REPEATABILITY

7.1 The repeatability of the results should be within the range of ± 5 percent.

INDIAN STANDARDS

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FOOD ANALYSIS

IS:

5398-1968 Methods for estimation of thiamine (vitamin B_1) in foodstuffs

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 Method for yeast and mould count in foodstuffs

5835-1970 Method for estimation of vitamin D in foodstuffs

5838-1970 Method for estimation of vitamin C in foodstuffs

5886-1970 Methods for estimation of carotenes and vitamin A (retinol) in foodstuffs

5887-1970 Method for detection of bacteria responsible for food poisoning and foodborne diseases

7219-1973 Method for determination of protein in foods and feeds

7234-1974 Method for estimation of folic acid in foodstuffs

7235-1974 Method for estimation of tocopherol (vitamin B) in foodstuffs

7530-1975 Method for estimation of pyridoxine (vitamin B_6) in foodstuffs

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