BHUTAN STANDARD

METHODS FOR ESTIMATION OF THIAMINE (VITAMIN B_1) IN FOODSTUFFS

UDC 664:543.867:577.164.11

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BTS 285: XXXX IS 5398: 1969

NATIONAL FOREWORD

This Bhutan Standard which is identical with IS 5398: 1969 METHODS FOR ESTIMATION OF THIAMINE (VITAMIN B1) 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 : 5398 - 1969
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Indian Standard

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BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002

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Indian Standard

METHODS FOR ESTIMATION OF THIAMINE (VITAMIN B_1) IN FOODSTUFFS

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Indian Standard

METHODS FOR ESTIMATION OF THIAMINE (VITAMIN B_1) IN FOODSTUFFS

0. FOREWORD

0.1 This Indian Standard was adopted by the Indian Standards Institution on 18 November 1969, 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 foodstuffs. Moreover, different methods of vitamin assay 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 microbiological methods, wherever applicable.

0.3 This standard covers two methods, namely, chemical method and microbiological method, used for estimation of thiamine in foodstuffs. Depending upon the available facilities and applicability, either of the methods may be used. It is desirable that the method used is stated in the test report.

0.4 In the preparation of this standard, considerable assistance has been derived from a number of standard books and publications. However, the methods included in this standard are predominantly those which have been tried in various laboratories in the country. Thus the methods prescribed in this standard are mainly based on practical experience within the country.

0.5 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*.

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1. SCOPE

1.1 This standard specifies chemical as well as microbiological methods for estimation of thiamine (Vitamin B_1) in foodstuffs.

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

2. QUALITY OF REAGENTS

2.1 Unless specified otherwise, pure chemicals shall be employed in tests and distilled water (*see* IS: 1070-1960*) shall be used when the use of water as a reagent is intended.

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

3. PREPARATION OF ASSAY SAMPLE

3.0 The technique used for preparing the material for the analysis is mostly 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, such as bread, biscuits and grains, should be ground to produce a fine powder.

3.2 Butter should be melted under constant stirring. Samples from margarine or cheese or other such foods should contain portions of the surface as well as of the interior.

3.3 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. CHEMICAL METHOD

4.0 Principle — Bound thiamine of foods is released by incubation with the enzymes takadiastase and papain at *p*H 4·2. The crude extract is purified by passing through a column of activated decalso (or its equivalent) and the thiamine in the eluate is estimated by oxidizing it to thiochrome and measuring the fluorescence. In case decalso (or its equivalent) is not available, lead acetate may be used, as it also gives almost comparable results.

4.1 Apparatus

4.1.1 *Chromatography Columns* — 25 × 1 cm.

4.1.2 *Centrifuge*

4.1.3 *Fluorometer*

4.1.4 *Blender*

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

4.2 Reagents

4.2.1 *Sodium Acetate Buffer* — 0·20 M (*p*H 4·2). Dissolve 34 g of sodium acetate ($CH_3COONa,3H_2O$) in 250 ml of water. To 30 ml of this solution, add 70 ml of 1 N acetic acid and dilute to 500 ml.

4.2.2 *Enzyme Solution* — Suspend 150 mg of takadiastase and 75 mg of papain in 5 ml of acetate buffer.

4.2.3 *Stock Thiamine Solution* — Dissolve 25 mg of pure dry crystalline thiamine hydrochloride in 250 ml of 0·01 N hydrochloric acid (*1 ml = 100 µg*).

4.2.4 *Activated Decalso or Equivalent* — Wash decalso (or its equivalent) which passes through 180- to 250-micron IS Sieve (*see* IS: 460-1962*) successively once with hot 3 percent acetic acid, once with hot potassium chloride solution (**4.2.7**) and again with hot 3 percent acetic acid followed by several washes with hot distilled water. Each washing consists of stirring decalso in the liquid for 15 minutes, allowing it to settle and then decanting. The final wash solution should be free of chlorides as tested with 1 percent silver nitrate solution. Store the decalso under water in a stoppered bottle. Decalso may also be stored in a dry state (dried at 100°C).

4.2.5 *Acetic Acid* — 3 percent (*v/v*).

4.2.6 *Acetic Acid* — 0·5 percent (*v/v*).

4.2.7 *Potassium Chloride Solution* — 25 percent in 0·1 N hydrochloric acid (*w/v*).

4.2.8 *Bromocresol Green Indicator* — 0·4 percent in 70 percent ethyl alcohol.

4.2.9 *Isobutyl Alcohol* — Redistilled (in an all glass apparatus), fraction distilling at 105° to 108°C collected and saturated with water.

4.2.10 *Anhydrous Sodium Sulphate*

4.2.11 *Potassium Hydroxide Solution* — 15 percent in water (*w/v*).

4.2.12 *Potassium Ferricyanide Solution* — 1 percent in water (*w/v*).

4.2.13 *Oxidizing Reagent* — Mix 1 part of potassium ferricyanide solution (**4.2.12**) with 9 parts of potassium hydroxide solution (**4.2.11**).

NOTE — This solution should be prepared fresh at the time of the assay.

4.2.14 *Blank Reagent* — Mix 1 part of water with 9 parts of potassium hydroxide solution (**4.2.11**).

*Specification for test sieves (*revised*).

4.3 Extraction — Cut the edible portion of the food into small pieces and homogenize well in a blender with a suitable volume of water. Weigh the total slurry and use a weighed aliquot (equivalent to 10 to 20 g of food) for extraction of thiamine. In case of dry foods, such as cereals, powder the material and use 10 to 20 g of the powder.

4.3.1 Take two aliquots of the slurry or powder into 250-ml flasks marked 'Test' and 'Recovery' and add 5 ml of enzyme suspension and 80 ml of acetate buffer to both. To the flask marked 'Recovery' add diluted thiamine standard solution equivalent to 25 µg of thiamine hydrochloride. Add 2 to 3 ml of toluene to both the flasks and incubate them at 37°C overnight. At the end of incubation period inactivate the enzymes by heating them in a boiling water-bath, make up the volume to 100 ml and centrifuge or filter the mixture to remove the residue.

4.4 Purification

4.4.0 Two methods have been described. The methods given under **4.4.2** and **4.4.2.1** may be used in case the decalso (or its equivalent) is not available.

4.4.1 Place small pieces of glass wool at the bottom of two chromatography columns (25 × 1 cm) (marked 'Test' and 'Recovery') and fill them uniformly with activated decalso, to a height of 8 cm. Wash the columns with 3 ml of 0·5 percent acetic acid. Take 20 ml each of test and recovery samples in beakers and adjust them to *p*H to 3·5 with 1 N hydrochloric acid. The end point may be checked in *p*H meter or with bromocresol green indicator (yellowish green colour).

4.4.1.1 Pour the samples and the washings on the respective columns and allow the liquid to drain. Wash the columns twice with 10 ml portions of boiling water and discard the eluates.

4.4.1.2 Eluate the thiamine from the columns successively with 10, 10 and 5 ml of boiling potassium chloride solution. Collect the eluates directly in a 25-ml volumetric flask or in a conical flask and make up the volumes to 25 ml after cooling.

4.4.2 *Basic Lead Acetate Solution* — Dissolve 180 g of lead acetate in about 700 ml of distilled water with the aid of heat. To the hot solution add 110 g of finely powdered lead-oxide (litharge) and continue boiling for 45 minutes with constant stirring. Cool the mixture, filter and dilute to 1 litre.

4.4.2.1 To 20 ml of the food extract, add 10 ml of the basic lead acetate solution, mix and centrifuge. To 20 ml of the supernatant, add 3 ml of 30 percent (*v*/*v*) sulphuric acid and 17 ml of water.

Remove the precipitate by centrifugation and store the supernatant solution at 5°C.

NOTE — If the lead acetate method is used, 30 μg of thiamine hydrochloride should be used for recovery, at the time of incubation with the enzyme (*see* **4.3.1**).

4.5 Procedure

4.5.1 *Conversion to Thiochrome* — Take two 5-ml aliquots (10 ml in the case of extracts obtained with the lead acetate method) from the purified extract marked ' Test ' into two separatory funnels (50 ml) or glass-stoppered test-tubes labelled ' Test ' and ' Blank '. In a third tube labelled ' Recovery ' take 5 ml of the purified extract marked ' Recovery ' (10 ml in case of extracts obtained with the lead acetate method). To the ' Blank ' add 1 ml of the blank reagent, followed within 1 minute by 15 ml of *iso*butanol. To the ' Test ' and ' Recovery ' tubes (or separatory funnels) add 1 ml of the oxidizing reagent, followed by 15 ml of *iso*butanol. Shake both the tubes (or separatory funnels) vigorously for 1 minute, allow the layers to separate and transfer the upper butanol layer containing thiochrome to another test-tube (use a 10-ml pippet with a rubber bulb to separate the epiphase if separatory funnels are not used). Add a small quantity of anhydrous sodium sulphate to remove the traces of water and read the fluorescence of the clear extracts in a fluorometer using suitable filters for thiamine estimation. A suitable reagent blank and a standard containing 2-5 μg of thiamine hydrochloride should also be prepared to balance the fluorometer.

4.6 Calculation — Calculate μg of thiamine per g of food sample (on dry basis or wet basis as the case may be) as follows:

$$\frac{\text{Test reading} - \text{Blank reading}}{\text{Recovery reading} - \text{Test reading}} \times \frac{\text{Dilution factor}}{\text{Weight of substance taken (or present in slurry)}}$$

NOTE — The dilution factor in the decalso method would be 25 and in the lead acetate method it would be 30.

5. MICROBIOLOGICAL METHOD

5.0 Principle — The micro-organism *Lactobacillus fermenti*-36; NCTC* No. 6991 (ATCC† No. 9338) has a specific requirement for its growth. The growth response on a defined medium complete in all respects

*National Collection of Type Cultures.
†American Type Culture Collection.

except for thiamine is proportional to the concentration of the vitamin added in the medium, up to a certain range.

5.1 Apparatus

5.1.0 The equipment listed in **5.1.1** to **5.1.21** are required. In addition, a nephelometer or a suitable photo-electric colorimeter is necessary for turbidimetric reading. Using *L. fermenti*-36, direct titration of lactic acid formed should not be employed as the organism responds to pyrimidine and thiazole moieties of the molecule after 18 hours of incubation.

5.1.1 *Incubator* — maintaining uniform temperature or water-bath (±0·5°C) in the range 30° to 37°C.

5.1.2 *Autoclave* — large enough to admit culture tubes in their racks and capable of accurate adjustments.

5.1.3 *Bacteriological Tubes* — rimless, of size 15×150 mm or 25×200 mm.

5.1.4 *Aluminium Caps* — to fit tubes in **5.1.3** (cotton plugs may be used if caps are not available).

5.1.5 *Culture Tube Racks* — to hold a total of 120 tubes. The racks should hold the tubes vertically and so designed as to permit free circulation of air. Metal racks, resistant to rust are required.

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

5.1.7 *Hypodermic Syringe* — 5 to 10 ml, for inoculating tubes.

5.1.8 *Refrigerator* — of sufficient capacity to hold reagents, basal medium and stock solution and culture.

5.1.9 *Electrically Operated Centrifuge* — to hold test-tubes.

5.1.10 *Sterilizing Can for Pipettes*

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

5.1.12 *pH Meter or Lovibond Comparator with Disc for Adjustment of pH*

5.1.13 *Erlenmeyer Flasks*

5.1.14 *Volumetric Flasks* — 100 ml.

5.1.15 *Volumetric Flasks* — 2-litres capacity.

5.1.16 *Filter Funnel* — 75 mm dia.

5.1.17 *Pipettes Graduated* — 5 ml.

5.1.18 *Pipettes Graduated* — 10 ml.

5.1.19 *Volumetric Pipettes* — 5 ml, 10 ml and 20 ml.

5.1.20 *Glass-Stoppered Cylinders or Bottles, Beakers* — as necessary.

5.1.21 *Automatic Dispenser Titrator* — if available.

5.2 Reagents

5.2.0 The principal reagent is a basal medium prepared by mixing a number of stock solutions in definite proportions. The stock solution may be preserved in the cold (refrigerator) in the dark with 0·1 percent chloroform and 0·5 percent toluene added to prevent microbial growth.

5.2.1 *Acid Hydrolyzed Vitamin-Free Casein*

5.2.1.1 *Extraction of casein* — Stir 100 g of vitamin-free casein with 250 ml of 95 percent ethyl alcohol for 15 minutes in a 1-litre beaker and filter with suction. Repeat extraction using 250-ml lots of the alcohol to remove any residual vitamin from the casein. Commercial brands of vitamin-free casein contain some B complex vitamins which may give high blanks if used without this treatment.

5.2.1.2 *Hydrolysis* — Transfer the alcohol-washed casein into round-bottom flasks of 1-litre capacity, preferably with 2 necks, ground to standard taper. Mix well with 500 ml of constant boiling hydrochloric acid. [A mixture of 1 volume of concentrated hydrochloric acid (37 percent) with 1 volume of water]. Fit the flask with water-cooled condenser and reflux over low flame or hot-plate for 8 to 12 hours. Since casein tends to froth during initial stages of hydrolysis, heat it carefully and gradually.

Mix the contents of the flask occasionally. After refluxing, remove as much hydrochloric acid as possible by distillation under vacuum or reduced pressure, and concentrate the hydrolysate to a thick paste. Air may be introduced through a bleeder tube placed well into the bottom of the flask to minimize bumping during the final stages of concentration. A temperature of 70° to 80°C is recommended. The acid concentration left should be low enough so that subsequent neutralization will not yield high salt concentration to retard bacterial growth on the basal medium. For this, it is usual to re-dissolve the paste in about 200 ml of water and repeat the distillation and concentration to remove additional hydrochloric acid.

5.2.1.3 *Adjustment of pH* — 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 the supernatant is light straw coloured. The decolourization may be complete in a few minutes to an hour depending on the charcoal used. This step also removes any residual niacin or folic acid that might have been carried through by the casein sample. Filter through a large fluted filter paper or by suction. Adjust the *p*H of the filtrate to 6·8, dilute to 1 litre, filter if necessary and store under toluene and over chloroform in the refrigerator.

Occasionally a precipitate may occur on standing. This is mainly tyrosine. This should be shaken up and the suspended material and fluid should be used. The insoluble material will dissolve when the entire medium is prepared.

5.2.2 *Cystine-Tryptophane Solution* — Suspend 4·0 g of *l*-cystine and 1·0 g *l*-tryptophane (or 2·0 g of *dl*-tryptophane) in 700-800 ml of water. Heat to 70° to 80°C and add 20 percent hydrochloric acid dropwise till the solids dissolve. About 12 ml of 20 percent hydrochloric acid will be required. Cool to room temperature and make up to 1 litre with water. Store as before.

5.2.3 *Adenine-Guanine-Uracil Solution* — Heat 0·1 g each of adenine sulphate, guanine hydrochloride and uracil in a 250-ml Erlenmeyer flask containing about 75 ml of water and 2 ml of concentrated hydrochloric acid. Cool when all solids have gone into solution. If a precipitate forms, add a few drops of concentrated hydrochloric acid and heat. Repeat until no precipitate forms on cooling and then transfer to a 100-ml volumetric flask and make up to volume.

5.2.4 *Thiamine-Free Yeast Supplement* — Dissolve 20 g of Difco or equivalent yeast extract in 200 ml of 0·5 N sodium hydroxide solution and autoclave for 30 minutes at 120° to 123°C. Neutralize the solution with glacial acetic acid and autoclave again for a further period of 10 minutes at 120° to 123°C to coagulate and precipitate any protein. Filter and adjust *p*H of solution to 1·5 with hydrochloric acid using thymol blue as external indicator. Shake the solution with 20 g of activated charcoal for 20 minutes, filter and adjust the *p*H of the filtrate to 1·5 if necessary. Shake the filtrate with 20 g of activated charcoal again for 20 minutes, filter and neutralize with sodium hydroxide and preserve under sulphur free toluene in a refrigerator. This solution will keep its activity for about 3 months.

5.2.5 *Photolyzed Peptone* — Dissolve 40 g of Difco/Bacto or equivalent peptone in 250 ml of water and 20 g of sodium hydroxide in 250 ml of water. Mix these two solutions. Allow to stand for 24 hours in the room. Expose the solution to the light of a 100-watt bulb kept at a distance of about 45 cm for 18 hours. At the end of the exposure, neutralize the solution with glacial acetic acid (25 to 28 ml) and add 14 g of anhydrous sodium acetate. Make up to 800 ml with water and preserve under sulphur-free toluene in the refrigerator. This solution generally keeps for 14 days.

5.2.6 *Salt Solution A* — Dissolve 25 g of dipotassium phosphate and 25 g of monopotassium phosphate and dilute to 500 ml with water. Store under toluene in the refrigerator.

5.2.7 *Salt Solution B* — Dissolve 10·0 g of magnesium sulphate ($MgSO_4$, $7H_2O$), 0·5 g of sodium chloride (NaCl), 0·5 g of ferrous sulphate ($FeSO_4$, $7H_2O$) and 0·5 g of manganese sulphate ($MnSO_4$, $4H_2O$) in water and dilute to 500 ml. Add 5 drops of concentrated hydrochloric acid and store the solution under toluene.

5.2.8 *Stock Thiamine Solution* — 1 000 µg/ml. Dissolve 100 mg of thiamine in a little water and make up to 100 ml with 2 percent (*v/v*) hydrochloric acid. Store the solution in the cold.

5.2.9 *Thiamine Solution Working Standard* — 0·02 µg/ml. The original stock solution generally keeps for several weeks if stored in the refrigerator. For various assays, first dilute 1 ml to 100 ml with water to get a solution of 10 µg/ml. From this solution dilute 5 ml to 1 000 ml to obtain a working standard solution of 0·02 µg/ml.

5.2.10 *Basal Medium* — The thiamine-free basal medium, sufficient for 100 tubes, shall have the following composition:

Photolyzed peptone	200 ml
Vitamin-free casein hydrolysate	25 ml
Cystine-tryptophan solution	25 ml
Glucose	20 g
Sodium acetate (hydrated)	10 g
Adenine + guanine + uracil solution	10 ml
Thiamine-free yeast supplement	10 ml
Sodium chloride	5 g
Inorganic salt solution A	5 ml
Inorganic salt solution B	5 ml
Water to make	500 ml

5.2.10.1 Mix the ingredients and adjust the *p*H to 6·5 with sodium hydroxide solution and make up the volume to 500 ml. If necessary, filter through a suitable sintered glass funnel or filter paper.

5.2.11 *Enriched Liver Tryptone Agar Medium for Stock Culture* — Mix, glucose 1·0 g, Difco/Bacto or equivalent tryptone 1·0 g, dipotassium phosphate 0·2 g, calcium carbonate 0·3 g, liver extract 10 ml, inorganic salt solutions (**5.2.6** and **5.2.7**) 0·5 ml each, thiamine 100 mg, agar Difco/Bacto or equivalent 1·5 to 2 g, in glass-distilled water and make up the volume to 100 ml. Steam to dissolve the agar, distribute aliquots into bacteriological tubes, plug with cotton and sterilize in an autoclave at 115°C for 10 minutes. Test for sterility by incubation of the tubes at 37°C for 24 to 48 hours and store in a refrigerator.

5.2.12 *Liver Extract for Reagent* (**5.2.11**) — Grind 10·5 kg of fresh liver and suspend in 2 litres of water. Heat on a steam-bath for 1 hour and filter through cheese cloth. Neutralize to *p*H 7·0, again heat for

15 minutes, filter through coarse filter paper and store in a dark bottle under toluene in the refrigerator.

5.2.13 *Culture Medium for Growing Inoculum* — Distribute 5 ml each of basal medium (reagent **5.2.10**) into clean and dry bacteriological tubes. Add 10 µg of thiamine to each. Make up to 10 ml with glass-distilled water and sterilize, after plugging with cotton, in an autoclave at 120° to 123°C for 10 minutes. Store in the refrigerator.

5.2.14 *Isotonic Salt Solution* — Dissolve 0·9 g of sodium chloride in 100 ml of water. Distribute 10 ml each into test-tubes, plug with cotton and sterilize at 120° to 123°C for 15 minutes.

5.3 Procedure

5.3.1 *Preparation of Stock Culture*

5.3.1.1 Prepare stab culture in 2 or more stock agar culture tubes using a pure culture of *L. fermenti*-36.

5.3.1.2 Incubate for 16 to 17 hours at 37·0° ± 0·5°C.

5.3.1.3 Store in the refrigerator for not longer than one week before transferring to the new stab.

5.3.2 *Preparation of Inoculum*

5.3.2.1 Transfer asceptically the cells from the stock culture into the liquid medium for inoculum (**5.2.13**) with the help of a sterile platinum needle.

5.3.2.2 Incubate for 16 to 17 hours at 37·0° ± 0·5°C.

5.3.2.3 Centrifuge this inoculum and pour off the supernatant asceptically.

5.3.2.4 Resuspend the cells in 10 ml of sterile saline (**5.2.14**), centrifuge again after mixing and pour off the supernatant.

5.3.2.5 Resuspend the cells into another 10 ml of sterile saline.

5.3.2.6 Dilute a few drops of this with 10 ml of sterile saline and use this diluted inoculum immediately for inoculating the assay tubes.

5.3.3 *Extraction of Samples for Assay*

5.3.3.1 Weigh sufficient amount of finely powdered/ground/homogenized material (to contain 5 to 10 µg of thiamine) into 120- or 125-ml

Erlenmeyer flask containing 25 ml of 0·1 N sulphuric acid (the amounts of acid may be increased, if necessary, if the volume of the material is large). Plug with cotton and steam for 30 minutes.

5.3.3.2 Cool, adjust the *p*H to 4·5 with 2·5 M sodium acetate buffer, using bromocresol green as external indicator.

5.3.3.3 Add 20 mg each of pepsin and takadiastase and mix well.

5.3.3.4 Incubate under a thin layer of sulphur-free toluene for 18 to 24 hours at 37°C.

5.3.3.5 Keep the digest in a steamer for 30 minutes at 100°C to stop enzymatic action and to drive off the toluene.

5.3.3.6 Cool and make up to 100 ml with water and filter through a Whatman No. 1 or equivalent filter paper. Keep it in the refrigerator.

5.3.4 *Preparation for Assay*

5.3.4.1 Take an aliquot of the sample extract, adjust *p*H to 6·5 with sodium hydroxide solution and make up to 50 ml with water, so that each millilitre of this extract contains approximately 0·015 µg of thiamine. This is called sample extract I.

5.3.4.2 Take another equal aliquot into a 100/125-ml Erlenmeyer flask and add 4·0 ml of freshly prepared 10 percent solution of crystalline sodium sulphite.

5.3.4.3 Mix and adjust the *p*H to 5·4 with 1 N sulphuric acid, using a suitable indicator externally.

5.3.4.4 Make up the volume to about 30 ml and autoclave at 120° to 123°C for 15 minutes.

5.3.4.5 Cool the autoclaved digest, oxidize excess sodium sulphite with a measured amount of 33 percent hydrogen peroxide using a freshly prepared mixture of equal parts of 5 percent potassium iodide solution, 1 percent starch solution and 50 percent sulphuric acid as external indicator.

5.3.4.6 Make up the volume to 50 ml. This sulphite treated extract is called sample extract II and serves to correct for any turbidity due to sample extract.

5.3.5 *Preparation of Assay Tubes*

5.3.5.1 Take 15 tubes of uniform size and number them serially.

5.3.5.2 Add 5 ml of basal medium into each of the 15 tubes.

5.3.5.3 Add 2 ml each of sample extract I to first five tubes.

5.3.5.4 Add 2 ml each of sample extract II to next ten tubes.

5.3.5.5 Add 3 ml of water into each of the first 6 tubes, that is, 5 tubes with sample extract I and one tube of sample extract II. The sixth tube serves as the blank for turbidimetric reading.

5.3.5.6 Add standard thiamine solution into the remaining 9 tubes at levels of 0·02 µg, 0·03 µg and 0·04 µg per tube, thus each level having been added in triplicate.

5.3.5.7 Make up the volume of these 9 tubes to 10 ml. These serve as the standard.

5.3.5.8 Plug with cotton or cap with aluminium caps and sterilize at 115°C for 10 minutes.

5.3.5.9 Cool the tubes after sterilization, and keep ready for incubation.

5.3.6 *Assay Procedure*

5.3.6.1 Bring the assay tubes (**5.3.5**) to 37°C.

5.3.6.2 Inoculate asceptically with a drop each of the diluted inoculum per tube.

5.3.6.3 Incubate for 17 to 18 hours at 37·0° ± 0·5°C.

5.3.6.4 Cool the tubes at the end of incubation period for 15 minutes at 4° to 5°C and measure the growth nephelometrically, using tube No. 6 as the blank.

5.4 Calculation

5.4.1 Plot a graph for each assay, plotting the average of three readings for each concentration against µg of thiamine used.

5.4.2 Determine the average value of the vitamin content of the replicates by interpolation from the readings.

5.4.3 Calculate µg thiamine/g food samples (on dry basis or wet basis, as the case may be) as follows:

$$\frac{\mu g \text{ of thiamine per 2 ml of extract I} \times 25}{\text{Aliquot of sample taken}} \times \frac{100}{\text{Weight of sample taken}}$$

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