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DRAFT TANZANIA STANDARD

General microbiological method for determination of amino acids in food and foodstuffs

TANZANIA BUREAU OF STANDARDS

0 Foreword

Amino acids as constituents of protein play an important role in the growth of all living organisms and thus their determination in foods is necessary.

Amino acids are evaluated in a large number of proteinous foods by the two methods, namely, chemical and microbiological analysis. The latter is simpler, less cumbersome and offer a reproducible result and is particularly usefully when an amino acids analyser is not available.

This method is intended to be used in order to eliminate differences which may arise between laboratories.

In preparation of this draft standards considerable assistance was derived from IS 7815-1975(reaffirmed 2015)-Methods for estimation of amino acids in foods, published by the Indian Standards Institution.

In reporting the results of a test or analysis made in accordance with this Tanzania standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with TZS 4.

1.0 Scope.

This draft Tanzania standard prescribes a microbiological method for estimation of amino acids in food and food stuffs.

2.0 References

The following referenced standards are indispensable for the application of this draft standard. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced standard (including any amendments) applies.

TZS 4, Rounding off numerical values

TZS 59, Distilled water quality-specification.

3.0 Principle

The method is based on the observation that certain micro-organisms require specific nutrients for growth. Using a basal medium complete in all respects except for the amino acid under test, growth responses of the organisms are compared quantitatively in standard and in unknown Solutions. Either the acid or the turbidity produced by the organisms is measured to determine the extent of growth and thereby the amount of nutrient in the test solution.

4.0 Quality of reagents

4.1 Unless otherwise specified, analytical grade chemicals and distilled water (see clause 2) shall be employed.

5.0 Microbiological assay of amino acids

5.1 Test organisms

5.1.1 *Leuconostoc mensenteroides* (ATCC No.8042)

This is used in the assay of all amino acids except threonine.

5.1.2 *Streptococcus faecalis* (ATCC No. 9790)

For use in assay of the threonine.

NOTE

Lactobacillus plantarum (ATCC No. 8014)

May also be used in assay of isoleucine, leucine, methionine, tryptophan and valine.

5.2 Preparation and maintenance of stock culture

Prepare stock culture of agar tubes and inoculate with pure culture (appropriate organisms to be used for different assays). Incubate the tubes for 16 to 24 hours at 37°C and store at 4°C. Transfer the culture into the new agar tubes every fortnight (two weeks).

5.3 Preparation of stock culture tubes

5.3.1 Salt solution A

Dissolve 25g each of dibasic potassium phosphate (KH_2PO_4) and monobasic potassium phosphate (K_2HPO_4) in water and make up to 250 ml with water.

5.3.2 Salt solution B

Dissolve the following salts in 250 ml of water to which are added a few drops of concentrated hydrochloric acid to obtain a clear solution. Store under toluene.

Magnesium sulphate ($MgSO_4 7H_2O$)	10.0g
Sodium chloride (NaCl)	0.5g
Ferric sulphate ($FeSO_4 7H_2O$)	0.5g
Manganese sulphate ($MnSO_4 H_2O$)	0.5g

5.3.3 Dissolve the following ingredients in 200ml of distilled water, adjust the pH to 6.8 and make the volume up to 250 ml.

Peptone	5.0g
Yeast extract	1.0g
Glucose, anhydrous	10.0g
Sodium acetate 3 H_2O	17.0g

Or

Sodium acetate, anhydrous	10.0g
Salt solution A	2.5 ml
Salt solution B	2.5 ml

Separately dissolve 7.5g of agar in 250ml distilled water by heating. Mix well both the solutions together, while the solution is still hot, transfer 10ml aliquots of solution into the test tubes. Plug with cotton, and

autoclave for 15 minutes at 0.082 MPa pressure. After cooling to room temperature store the tubes at 2 to 4°C and for maintaining stock cultures.

5.4 Preparation of inoculum broth

Prepare the inoculum broth in the same way as the culture medium (see 5.2) excepting, that instead of the agar solution, add more distilled water after adjusting the pH to 6.8 and make up the volume to 500 ml. Transfer 10 ml- aliquots of the solution into the test tubes, then plug with cotton, autoclave for 15 minutes at 0.082 MPa pressure, cool at room temperature and store at 2 to 4°c.

5.5 Preparation of inoculum

A day prior to use, inoculate inoculum broth tube with a loopful of culture from the stock culture and incubate at 37°C for 16 to 18 hours. Take a centrifuge tube plugged with cotton, an all glass syringe, a 0.8 mm needle, and some saline (0.9%) in a conical flask. Sterilize the saline by autoclaving for 15 minutes at 0.103 MPa pressure. For syringes, needles and centrifuge tubes by placing in a hot air-oven at 160°C for one hour. Transfer the cells from the inoculum tubes to the centrifuge tubes and centrifuge, decant off the supernatant and resuspend the cells in sterile saline and centrifuge. Repeat the process two or three times. Take the resuspended cells in sterile syringe and inoculate the assay tubes with one drop each of the inoculum. The optical density of the inoculum should be about 0.1.

6.0 Preparation of solution for assay tubes

6.1 Salt solution A

Dissolve 12g each dibasic potassium phosphate (KH_2PO_4) and monobasic potassium (K_2HPO_4) in water and make up to 100ml with water.

6.2 Salt solution B

Mix and dissolve the following in water to make 100 ml.

Magnessium sulphate ($MgSO_4.7H_2O$)	4.0g
Manganese sulphate ($MnSO_4.4H_2O$)	0.4g
Ferric sulphate ($FeSO_4.7H_2O$)	0.2g
Sodium chloride (NaCl)	0.2g

6.3 Adenine Guanine-Uracil(AGU) solution

Dissolve 0.2g of (AGU) in water using concentrated hydrochloric acid drop wise for dissolving and make up to 100ml with water.

6.4 Xanthine solution

Dissolve 0.2g of xanthine in water using liquor ammonia drop-wise for dissolving and make up to 100ml with water.

6.5 Vitamin solution

Dissolve the following in water and make up to 100ml.

Para-amino benzoic acid (PABA)	*2.00g*
Biotin	*0.02mg*
Ca-panthothenate	*10.00mg*
Folic acid	*0.2mg*
Niacin	*20.00mg*
Pyridoxal HCL	*6.00mg*
Pyridoxalamine 2 HCL	*20.00mg*
Pyriodoxine HCL	*10.00mg*
Riboflavin (see note)	*10.00mg*
Thiamine	*10.00mg*

NOTE: Riboflavin is weighed separately, dissolved in a few milliliters of acetic acid and mixed with other solution.

6.6 Non-essential amino acid solution

Dissolve the following amino acids in water using concentrated hydrochloric acid drop wise dissolving and make up to 100 ml

dl-alanine	*4.0g*
l-asperagine acid	*8.0g*
l-aspertic acid	*2.0g*
l-glutamic acid	*1.0g*
l-cystine	*6.0g*
Glycine	*2.0g*
l-proline	*2.0g*
dl-serine	*1.0g*
l-tyrosine	*2.0g*

6.7 Essential Amino Acids g/100ml

l-arginine HCL	*4.85*
l-histidine HCL	*1.24*
dl-Isoleucine	*5.00*
l-leucine	*2.50*
l-lysine HCL	*5.00*
dl-phenylalanine	*2.00*
dl-threonine	*4.00*
dl-tryptophan	*0.80*
dl-valine	*5.00*
dl-methionine	*2.00*

Dissolve each of the following amino acids in water separately using hydrochloric acid drop-wise for dissolving and make up to 100ml with water keep each amino acid solution as a separate bottle

6.8 Composition of Basal Medium

6.8.1 For Leuconostoc Mesenteroides

Glucose	*5.0g*
Sodium acetate	*4.0g*
Ammonium chloride	*0.6g*
Salt solution A	*1.0 ml*
Salt solution B	*1.0ml*
AGU solution	*1.0ml*
Xanthine solution	*1.0ml*
Vitamin solution	*1.0ml*
Non-essential amino acid solution	*1.0ml*
l-arginine HCL	*1.0ml*
l-histidine	*1.0ml*
dl-isoleucine	*1.0ml*
l-lysine HCL	*1.0ml*
dl-phenylalanine	*1.0ml*
dl-threonine	*1.0ml*
dl-tryptophan	*1.0ml*
dl-valine	*1.0ml*
dl-methionine	*1.0ml*
l-leucine	*1.0ml*

Mix the solution well, adjust the pH 6.8 and make the volume up to 100ml with water.

6.8.2 For *Streptococcus faecalis*

Hydrogen peroxide (H_2O_2)	*75ml*
Treated peptone	*100mg*
l-methionine	*100mg*
l-cystine	*100mg*
l-tyrosine	*200mg*
Glycine	*100mg*
Glucose (anhydrous)	*20g*
Sodium acetate (hydrated)	*33g*
Ammonium chloride	*6g*
Ammonium chloride	*6g*
AGU solution	*12ml*
Xanthine solution	*12ml*
Salt solution A	*5ml*
Salt solution B	*5ml*

Vitamin Addition

Aneurine	*1000µg*
Calcium pantonthenate	*100µg*
Nicotinic acid	*2000µg*
Riboflavin	*2000µg*
Pyrodoxine HCL	*1600µg*
p-amino benzoic acid(PABA)	*50µg*
Water to make	*500ml*

Mix the solution well, adjust the pH to 6.8

NOTE-In preparation of basal medium, the amino acid to be assayed is omitted.

6.9 Preparation of stock standards solution

Use for the preparation of standard solutions1-form of amino acids. Dissolve 50mg of amino acid in 100ml of water to make 500µg/ml. A few drops of concentrated hydrochloric acid may be needed to dissolve some of the amino acids. The standards stock solution should be prepared fresh every three months.

6.10 Working standard range

Amino acid	Range (µg)	Dilutions(ml)
Arginine	0 to 40	4 to 50
Histidine	0 to 10	2 to 100
Isoleucine	0 to 25	2.5 to 50
Leucine	0 to 25	5 to 100
Valine	0 to 25	1 to 50
Methionine	0 to 10	1 to 50
Phenylalanine	0 to 10	2 to 50
Threonine	0 to 20	1 to 100
Tryptophan	0 to 10	1 to 50
Lysine	0 to 30	3 to 50
Cystine	0 to 5	1 to 100
tyrosine	0 to 10	2 to 100

NOTE- Make the dilutions from a stock standards solution containing 500 µg amino acid per ml.

6.11 Standards levels

Take 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, and 1.0 ml in triplicate, add enough water to make 1ml and followed by 1ml of basal medium. Cover the tubes with cotton wool plugs and wrap with brown (Kraft) paper to protect the cotton wool plugs from getting wet during autoclaving. Alternatively use aluminum caps.

6.12 Preparation of samples

6.12.1 Acid hydrolysis- Add 1g of the sample to 25 ml of 2.5M hydrochloric acid. Autoclave the mixture for 6 hours at 0.103MPa pressure. Cool and add 2 ml of 2.5 ml sodium acetate. Adjust the pH to 4.5 and Make the solution up to a known volume and filter and note the known volume. Take an aliquot and adjust the pH to 6.8 with sodium hydroxide, and dilute to the required concentration.

6.12.2 Alkali hydrolysis for estimation of tryptophan and tyrosine only

Add 1g of the sample to 25ml of 2mol/l sodium hydroxide and autoclave the mixture for 6 hours or add 5g of barium oxide to 25 ml of water and autoclave for 8 hours at 0.103 MPa pressure. Then cool to room temperature, adjust the pH to 4.0 with either hydrochloric acid or acetic acid and make up to a known volume and filter. Adjust pH of the aliquot for 6.8 and dilute to get the required concentration. Racemization takes place during this treatment and the amino acids will all be in dl-form.

6.12.3 Sample should be preserved and stored under toluene at 4^{o}C and used for analysis preferably within a month's 'time.

6.13 Sample levels

Take 0.2, 0.4, 0.6, 0.8, and 1.0 ml in duplicate and enough water to make to1ml and followed by 1 ml of basal medium. Cover the tubes with cotton plugs and wrap with brown (kraft) paper to protect the cotton plug from getting wet during autoclaving. Alternatively use aluminum caps.

7.0 Procedure

7.1 Standard tubes

Sterilize the tubes of basal medium containing standards levels of amino acids as in 6.11 by autoclaving at 120^{o}C for 10 minutes. Cool to room temperature and inoculate 3 tubes of each standard with inoculum as in 5.5. For acidimetric titration method see (7.3), incubate the inoculated tubes at 37^{o}C for 72 hours, except for cysteine where it should be done for 18 to 24 hours. If turbid metric method see (7.4) is used, incubate the inoculated tubes at 37^{o}C for 16 to 20 hours.

7.2 Sample tubes

Repeat the procedure as 7.1 for sample tubes using the same incubator at 37^{o}C7.3 Acidimetric method

7.3.1 0.02 mol/l sodium hydroxide and 0.1 per cent bromothymol blue indicator solution.

7.3.2 Transfer the content quantitatively to a 150ml conical flask, rinsing the tubes with distilled water twice. Add about 0.2 ml of 0.1 percent bromothymol blue indicator and titrate the solution against 0.02 mol/l sodium hydroxide to an end point of greenish blue color around pH 6.8. a pH meter may be used in place of indicator solution.

7.3.3 Calculation

Draw a standard curve for the assay by plotting the volume of 0.02mol/l sodium hydroxide on the Y-axis against concentration of the amino acid per tube in the standard series on the X-axis. It is preferably to plot these on logarithmic scale in order to obtain a straight line of the curve where the straight line of the curve can be utilized to determine amino acid content in the sample tubes. Determine amino acid content of the tubes in unknown series by interpolation of the titre values on the standard curve. Calculate the

average for 1 ml of test solution from values obtained from not less than three tubes which do not vary by more than 10 percent of the average. Calculate the amino acids content of the test solution using the following relationship:

$$\mu g\ of\ test\ amino\ acid/g = \frac{Average\ \mu g\ per\ ml\ x\ volume\ \ x\ Dilution\ factor}{Mass\ of\ the\ sample}$$

7.4 Turbid metric method

7.4.1 Apparatus-Nephelometer (turbid meter)

7.4.2 Method

Take nephelometric reading of the growth using the tube supplied with the nephelometer. Transfer the same known volumes of the growths in the standard tubes, commencing with the lowest amount of amino acid, that is, the highest dilution made. Draw a standard curve for the assay by plotting the turbid metric reading s on the Y-axis against concentration o the X-axis. It is preferable to plot these on logarithmic scale in order to obtain a straight line or a type of curve where the straight line of the curve can be utilized to determine the amino acid content of the sample tubes by interpolation of the turbidimetric values on the standard curve. Calculate the average for 1ml of test solution from the values obtained for the less than three tubes which do not vary by more than 10% of the average. Calculate amino acid content of the test solution as in 7.3.3.

NOTE-It is essential that the standard curves as in 7.3.3 and 7.4.2 be constructed each time that an assay is undertaken since conditions of autoclaving, temperature of the incubation etc., which influence the standard curve reading, cannot be duplicated exactly from time to time.

8 Repeatability

The method should be repeatable within the range of10.