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DRAFT TANZANIA STANDARD

Determination of vitamin E (tocopherol) in food and food stuffs-Part 1-General method

TANZANIA BUREAU OF STANDARDS

0 Foreword

Vitamin E(tocopherol) is one of the important growth factors required in the diet of human being at specific requirement level.It is widely distributed as a fat soluble substance in variety of seeds, germ and leaf vegetables.

In view of its wide distribution in foods, deficiency of the vitamin is unlikely to occur. However there existsa need to prepare this draft Tanzania standards in order to quantify the vitamin in processed food and food stuffs.

In preparation of this draft Tanzania standards assistance was drawn from

IS 7235:1974(reaffirmed 2015), Method For Estimation Of Tocopherols (vitamin E) In Foodstuffs published by Indian Bureau of Standards.

In reporting the results of a test or analysis made in accordance with this standards, if the final value observed or calculated is to be rounded off, it shall be done in accordance with TZS 4: (see clause 2).

1.0 Scope

This draft Tanzania standards prescribes a chemical method for determination of Vitamin E(tocopherol) in food and foodstuffs.

2.0 References

For the purpose of this draft Tanzania standard the following references shall apply where by for dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies

TZS 59,*Water-distilled quality- Specification.*

TZS 4:, *Rounding off numerical values.*

3.0 Forms of tocopherol

The distinct forms of tocopherol that exist in nature and which have Vitamin E activity are given below:

Structural name	*Common name*
5,7,8-trimethyl tocol	α - tocopherol (alpha)
5,8-dimethyl tocol	β - tocopherol (beta)
7,8-dimethyl tocol	γ - tocopherol (gamma)
8-ethyl tocol	δ - tocopherol (delta)
7-methyl tocol	λ - tocopherol (lambda)
5-methyl tocol	ε - tocopherol (epsilon)

4.0 Principle

4.1 α -tocopherol- The extract containing total tocopherol is purified for interfering substances, such a vitamin A and carotenoid by reduction with zinc and treatment with antimony trichloride, and fats are elliminared by saponification. α - and ε -tocopherol are separated together from the other tocopherol by

chromatography on alumina and determined colorimetrically with iron dipyridyl. Calculation is carried out with the aid of a calibration curve and a recovery test.

4.2 Total tocopherol- The β -, γ -, δ -, λ - and ε - tocopherol still adsorbed on the alumina are consequently eluted together and determined colorimetrically(clause 4.1) The sum of the results obtained by both assays gives the total tocopherol content.

5.0 Apparatus

5.1Chromatographic column-

5.2 Spectrophotometer- For taking reading at 515 nm

5.3 Blender

5.4 Stop watch

6.0 Reagents

6.1 Quality of reagents: Unless specified otherwise, analytical grade chemicals, and distilled water conforming to TZS 59:1980(see clause 2) shall be employed.

6.2 Methanol- Distil 10 litres of methanol over 5 g of potassium permanganate and 10 g of potassium hydroxide.

6.3 Petroleum ether- Boiling point 30°C to 40°C or 60°C to 80°C or 90°C to 115°C distilled over concentrated sulphuric acid.

6.4 Ethanol- Absolute, purified like methanol(6.2) and distilled repeatedly until no fluorescence can be detected in ultra- violet light.

6.5 Diethyl ether- Free from peroxide, treated with ferrous sulphate

6.6 Elution mixtures

6.6.1 Mixture of 100 ml of diethyl ether(6.5) and 100 ml of petroleum ether(6.3)

6.6.2 Mixture of 99 ml of cyclohexane (6.17) and 1 ml of diethyl ether (6.5).

6.6.3 Mixture of 15 ml of diethyl ether(6.5) and 85 ml of petroleum ether(6.3).

6.7 Sodium ascorbate- Pure, powder.

6.8 Methanolic potassium hydroxide-Approximately 1 M, prepared shortly before use.

6.9 Aluminium oxide for chromatography degree of activity 1with mesh size such that 50% is able to pass through a number 100 sieve and absorption index 30-40.

Activate aluminium oxide powder by heating at 400°C for 2-4 hours and allow to cool while being protected from moisture. Shake 100 g of the powder with 10 ml to 15 ml of water till all clots disappear and the powder flows freely. The deactivated alumina is stable for one day and should be prepared at-least one hour before use.

NOTE- It is essential to standardize the activity of each alumina. The test is carried out with vitamin A which is added to a column and a column is developed by hexane. Vitamin A is located either by antimony chloride reagent or under ultra-violet light. Suitability of alumina should be checked with pure α -tocopherol and with β, or γ - tocopherols.

6.10 Ferric chloride- 0.2 per cent alcohol solution. Dissolve 0.2 g of ferric chloride in 100 ml of ethanol (6.4). The solution is sensitive to light. It should be prepared shortly before use and stored in brown bottle and kept away from light.

6.11 α,α'- Dipyridly-0.5 per cent- Alcoholic solution 0.5g α,α'-Dipyridly is dissolved in 100 ml of ethanol(6.4).

6.12 Antimony trichloride- Add 22 g of antimony trichloride to 100 ml of ferric chloride and reflux until complete dissolution is achieved. When cold, keep the reagent in brown bottle over highly activated aluminium oxide. Agitate and filter before use.

6.13.Hydrochloric acid

6.13.1 *Hydrochloric acid*, concentrated- 38 per cent.

6.13.2 *Hydrochloric acid*,- Approximately 1 M. Add 94 ml of concentrated hydrochloric acid to 1 litre of distilled water

6.14 Zinc- powder, pure

6.15 Ammonium hydroxide solution- 10 per cent.

6.16 Enzymes

6.16.1 *Lipase*

6.16.2 *Clarase 900*

6.16.3 *Pig pancreatic powder*

6.17 Cyclohexane-Pure, distilled.

6.18 Water- The distilled water used for washing the dimethyl ether petroleum ether extracts should be saturated with a mixture of equal parts of the two solvents.

6.19 Vitamin E test solution- Rapidly weigh a known amount (90 mg to 100 mg)of pure α,α'- tocopherol in a 100 – ml volumetric flask. Immediately dissolve in ethanol (6.4), and make up the solution to volume with the same solvent. The solution can be kept for 5 to 10 days at 4^{o}C in the dark.

Dilute ten times with ethanol before use.

7.0 Preparation of assay sample

7.1 It shall be ensured that the sample taken for the assay is representative of the submitted materials and that deterioration of the vitamin to be examined is minimized. All samples shall be stored in the dark at 4^{o}C.

7.2 Powder and liquids shall be mixed thoroughly until homogeinity is achieved.Dry materials such as bread, biscuits and rice shall ground and mixed thoroughly.

7.3 Fats shall be melted under constant stirring. Samples shall contain portions of the surface as well as of the interior.

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

8.0 Procedure for the extraction of vitamin E from preparations containing the vitamin in water dispersed form.

8.1 Vitamin E is extracted from these products according to the Roese-Gottlieb procedure as follows:

40 ml of the liquid is mixed in 250 ml glass-stoppered cylinder with the following reagents and shaken thoroughly after each addition:

10 ml of 10 per cent ammonia solution;

40 ml of ethanol, absolute;

80 ml of diethly ether;

80 ml of petroleum ether.

The mixture is let to stand for at-least 15 minutes in the dark.When complete separation of the layers is achieved, the total volume of the petroleum ether solution is determined, and an aliquot thereof is evaporated carefully, and the residue further treated as described in 8.2.

8.2 An aliqout of the ether petroleum ether extract containing at-least 100 micrograms of vitamin E is evaporated in vacuum and the residue, depending on its content of fat, is further treated as described in 9.4.1 or 9.3.3.

9.0 Procedure

9.1 General

The determination of vitamin E is carried out by colorimetry. Vitamin E is oxidized by ferric chloride and ferrous chloride so formed, gives, on reaction with $\alpha\ \alpha'$ -dipyridly, a red coloured solution which is measured at 515 nm.

9.2 The procedure allows separation and estimation of α and β tocopherol content.

9.3 Calibration Curve - Measure increasing amounts (0 to 200 micrograms of vitamin E) of the test solution (6.19) each into a l0-ml volumetric flask and dilute with ethanol up to a volume of 8 ml. Add 1 ml of α, α'-dipyridyl solution (6.11) and 1 ml of ferric chloride solution (6.10) consecutively to the flask. Immediately on the addition of the ferric chloride solution, set the stop-watch in motion. Set the calorimeter to the zero point with ethanol (6.4) and after two minutes measure the absorption of the vitamin E solution at 515 nm.

NOTE- It is essential that the calibration curve runs concurrently with the sample. The procedure should be carried out in non-actinic light.

9.4 Extraction of Tocopherols from Foods

9.4.1 Dry products with low total lipid content - Weigh accurately 10 g (if a larger sample is needed, the quantity of the solvents should be increased accordingly) of sample, containing at least 1 mg of vitamin E, in a non-actinic glass vessel. After addition of about 100 mg each of sodium ascorbate (6.7) and of the three enzymes (6.16), stir the sample into a paste with 35 ml of warm (70°C) distilled water and let the flask stand for 20 minutes on a water-bath at 45°C in the dark. Then agitate the mixture with 6'5 ml of ammonia (6.15) and with 35 ml of ethanol (6.4) and cool to room temperature. Shake thoroughly with 70 ml of diethyl ether (6.5) followed by gentle agitation with 70 ml of petroleum ether (6.3), and allow to stand until separation of the layers occurs. Separate the layers. Remove the alcohol contained in petroleum ether layer by washing approximately 100 ml of the later twice each time with 10 ml of water (6.18). Measure an aliquot of the washed extract containing at least 200 micrograms of total tocopherol into each of two round-bottomed flasks. To one of the flasks add an amount of standard vitamin E solution (6.19) corresponding to its expected α- tocopherol content.

9.4.2 ***Dry products containing lipids***- Extract the foods as given in 5.6.2.1 and evaporate the extract in the recovery flask in vacuum or under nitrogen and saponify the samples (see 9.4).

9.4.3 ***Fats and oils*** -Weigh exactly (butter, etc) 5 to 10 g of sample in a 100 ml volumetric flask, dissolve in petroleum ether (6.3) and bring up to volume with the same solvent. Measure an aliquot of the solution, corresponding to a total tocopherol content of at least 100 micrograms and to a fat amount not exceeding 3 g (if a larger amount of fat is needed, the quantity of reagents and solvents necessary for further treatment should be increased accordingly), twice into two separate round-bottomed flasks and treat with about 100 mg of sodium ascorbate (6.7). In one of the flasks (Recovery Test), add an amount of vitamin E test solution (6.19) corresponding to the α-tocopherol content of the assay solution. Subsequently, treat both normal and recovery tests in the same way. Evaporate the solvent in a vacuum and saponify the residue (see 9.4.4).

NOTE - Peroxides eventually present in fats may destroy the vitamin E during the assay and should be removed as follows: Take up the residue in 10 ml of elution mixture (6.6.3), filter the solution through a 5-cm high column of alumina (6.9), and wash out total tocopherol out of the column with 80 ml of elution mixture (6.4.3). Saponify the residue obtained after evaporation of the filtrate in vacuum and treat further as described in 9.4.3.

9.4.4 ***Saponification*** - Add 15 ml of methanolic potassium hydroxide solution (6.8) to the residue obtained from 9.4.2 and 9.4.3 and add 100 to 200 mg sodium ascorbate powder (6.7). Reflux for 15 minutes in a water-bath at 60 to 70°C. The mixture should be stirred from time to time (a magnetic stirring is suitable)

9.4.5 ***Extraction of the unsaponifiable matter*** - Cool the soap solution to approximately 40°C and transfer quantitatively into a 250-ml glass-stoppered cylinder with 50 ml of methanol (6.2). Then cool it to room temperature and extract by thoroughly shaking it with 100 ml of petroleum ether (6.3). As soon as the layers have separated add 10 ml of distilled water and repeat the shaking. (Only after the addition of water, the quantitative separation of the methanol-petroleum ether mixture is achieved.) Remove the aqueous layer by suction and wash the extract first with 20 ml of distilled water, then, with 20 ml of hydrochloric acid (6.13.2).

9.4.6 ***Purification of the extract*** - The extraction obtained directly from dry products containing **low** am

ount of lipids (9.4) of extract of unsaponifiable matter is evaporated in vacuum or under nitrogen. Mix the residue thoroughly with 3 ml of antimony trichloride (6.12). After 5 minutes, add 10 ml of ethanol (6.4), 10 ml of concentrated hydrochloric acid (6.13.1) and about 50 mg of zinc powder (6.14). Repeat the addition of zinc powder two or three times until the solution becomes colourless. (During this operation, the flask should be cooled in order to prevent overheating of the mixture above 40°C.) After this treatment

quantitatively transfer the mixture to a 250-ml glass-stoppered cylinder with 30 ml of ethanol (6.4) and thoroughly shake with 50 ml of diethyl ether (6.3). Then agitate it with 50 ml of petroleum ether (6.3) and, after the layers have separated, with 50 ml of distilled water. The impurities (products formed after reaction with antimony trichloride and some zinc powder) contained in the extract should be removed before the assay is continued. Therefore, after separation of the layers has occurred filter an aliquot of the ether-petroleum ether extract corresponding to 50 to 150 micrograms of total tocopherol, through a 5-cm high column of alumina (6.9) previously soaked with eluting mixture (6.6.1). Wash out total tocopherol of the column with 80 ml of eluting mixture (6.6.1).

9.5 Determination of α - tocopherol.

9.5.1*Chromatography* -Evaporate the filtrate obtained under 9.3.3 or an aliquot containing 50 to 100 micrograms of total tocopherol in vacuum and take up the residue in 20 ml of cyclohexane 6.17). Fill up the chromatographic tube to a height of about 12 cm by pouring slurry of alumina in cyclohexane (6.17). Add cyclohexane (6.17) through until it covers the alumina for about 1 cm. The cyclohexane solution of the sample is adsorbed on the alumina and washed with 60 ml of cyclohexane (6.17). Replace this collecting flask with a clean one and elute the fraction containing α- and β -tocopherol with 130 ml of elution mixture (6.6.2). The other tocopherols remain adsorbed on the alumina (see 9.3).

9.5.2 *Colour reaction and measurement* - Evaporate the elute under reduced pressure or in a stream of nitrogen and take up the residue in 8 ml of ethanol (6.4). Carry out the addition of 1 ml of α,α'- dipyridyl (6.11) and 1 ml of ferric chloride solution (6.10) and measure the absorption exactly as described in 9.3.

9.5.3 *Calculation*

The calibration curve obtained by measuring the prescribed amounts of α-tocopherol is linear but does not pass through the origin, Therefore the vitamin E content cannot be calculated directly from extinction readings found for the normal and the recovery tests.

Micrograms of vitamin E/ 100 g of sample = $\frac{100\times c\times b}{a-b}$

Whereby

c = amount in micrograms of vitamin E added in the recovery test per gram of sample,

b = micrograms of vitamin E per gram of sample found in the normal test by means of the calibration curve, and

a = micrograms of vitamin E per gram of sample found in the recovery test by means of the calibration curve.

9.6 Accuracy - The accuracy of the method is $\pm$ 5 percent.

9.7 Sensitivity - 10 micrograms vitamin E/g.

NOTE - The recovery test may be omitted when products of the same type have to be assayed frequently, provided that the loss of vitamin E during the whole assay is previously determined and does not exceed 7 percent**.**

10.0 Determination of Total Tocopherol

The total tocopherol content can be determined by continuing then chromatography after elution of the α and β fraction. The collecting flask is changed and the other tocopherols are eluted with 80 ml of elution mixture (6.6.2). This eluate, which contains β -, γ -, δ -, λ - and ε - -tocopherol, is evaporated in vacuum and the residue is taken up in 8 ml of ethanol (6.4) and treated with ferric dipyridyl as described in 8.1. However, measurement of the absorption is carried out 10 minutes after addition of the ferric chloride solution because the tocopherols mentioned above react more slowly than α -tocopherol with ferric dipyridyl. Calculated by reference to the calibration the content of these tocopherols is curve obtained with α -tocopherol.

10.1 Calculate the total tocopherol content by adding this result to the one obtained for the α - and β - fraction.