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DRAFT TANZANIA STANDARD

Determination of vitamin D in foods and food stuffs-Part 1-General method

TANZANIA BUREAU OF STANDARDS

0 Foreword

Vitamin D is one of the important growth factors required in human body at specific requirement level. Foods known to contain vitamin D are milk and milk products,eggs, fox,and pigs liver. Fish oil have the largest vitamin D contents. Also a number of foods contain a considerable amount of the D provitamins, especially milk and milk products,eggs and fungi. Deficiency of the vitamin D is very unlikely to occur given that is synthesized by the action of sunlight on the skin.

The development of this Tanzania standard was necessitated by the need to quantify the vitamin in foods and food stuffs.

In the preparation of this standard assistance was drawn from IS 5835:1970 (reaffirmed 2015) -Method For Estimation Of Vitamin D In Foodstuffs, Published by the Bureau of Indian Standards

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(see clause 2).

1.0 Scope

This Tanzania Standard prescribes a method for determination of vitamin D in food and foodstuffs. The distinct forms of vitamin D exist in nature and which have vitamin D activity are vitamin D2(ergocalciferol) and vitamin D3 (cholecalciferol).

2.0 References

For the purpose of this 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 for analytical laboratory use – Specification and test method*

TZS 4 *Rounding off numerical values*.

3.0 Principle

The method is based upon the measurement of colour produced when a solution of vitamin D is treated with antimony trichloride.

4.0 Apparatus

4.1 Chromatographic tubes

4.1.1 First tube

Arrange for descending column chromatography a tube of 2.5 cm (inside) diameter, about 25 cm long, and constricted to 8 mm diameter for a distance of 5 cm at the lower end, by inserting at the point of constriction a sintered-glass disc of coarse porosity or a small plug of glass- wool. The constricted portion may be fitted with an inert plastic stopcork.

4.1.2 Second tube- Select a tube that is made up of three sections:

a) a flared top section, 18 mm inside diameter and approximately 14 cm long and

b) a middle section,6 mm inside diameter and approximately 25 cm long. insert a small plug of wool in the upper 1 cm portion of the constricted section.

4.2 Chromatographic columns

4.2.1 First column – To about 125 ml of iso-octane contained in screw-capped wide mouth bottle, add 25 g of chromatographic siliceous earth, and shake with a mechanical shaker until a slurry is formed. Add, drop wise and with vigorous mixing, 10 ml of polyethylene glycol 600. Replace the bottle cover and shake vigorously for 2 minutes. Pour about half of the resulting slurry into the chromatographic tube and allow it to settle by gravity. Then apply gently sunction and add the remainder of the slurry in small portions parking each with portion with 20 mm disc plunger. When a solid surface has formed removed the vaccum, and add about 2 ml of iso-octane.

4.2.2 Second column - Pack the midsection of the tube with 3 g of moderate coarse chromatographic fuller's or equivalent earth with the aid of gentle sunction (about 125 mmHg.)

5.0 Reagents

5.1 Quality of reagents: Unless specified otherwise analytical grade chemicals, and distilled water conforming to TZS59 (see clause 2) shall be employed.

5.2 Chromatographic fuller's or equivalent earth

Use chromatographic fuller's or equivalent earth having a water content corresponding to not less than 8.5 per cent and 9.0 per cent inclusive of loss on drying.

5.3 Solvent hexane- Redistilled, if necessary, so that when measured in a 1 cm cell at 300 mm with a suitable spectrophotometer, against air as the blank, the absorbances shall not be more than 0.070nm.

5.4 Ethylene dichloride- Purified by passage through a column of granular (passing through 75-710 micron sieve) silica gel (chromatographic grade).

5.5 Potassium hydroxide solution

Dissolve 780 g of potassium hydroxide in water to make 1000 ml.

5.6 Cotton seed oil- Saponify 10 g of the oil and dissolve the unsaponified residues in 10 ml of solvent hexane. In a separate container place 0.4 ml of ferric chloride solution (1 in 1000 and 12 ml of 1 in 6000 solution dipyridyl in absolute alcohol) then mix, and 5 minutes later read the absorbency in a 1 cm cell at 520 nm, with a suitable spectrophotometer using absolute alcohol as the blank. Then add 0.2 ml of solvent hexane solution of the unsaponifiable residue, and after 5 minutes read the absorbance. The difference between the first and the second absorbance shall be not less than 0.125 nm.

5.7 Sodium sulphate- Anhydrous.

5.8 Sodium sulphate –Decahydrate.

5.9 Colour reagent-Prepare the two solution as follow:

Solution A

Empty without weighing, the entire contents of previously unopened 133 g bottle of dry, crystalline antimony trichloride into a flask containing about 400 ml of ethylene dichloride. Add about 2 g anhydrous alumina, mix and filter through filter paper into a clear-glass-stopperd container calibrated at 500 ml. Add ethylene dichloride to make the volume 500 ml, and mix. The absorbance of the solution measured in a 20 mm cell at 500 nm with a suitable spectrophotometer, against ethylene dichloride, shall not exceed 0.070.

Solution B

Mix, under a hood, 100 ml of acetyl chloride and 400 ml of ethylene dichloride.

Mix 45 ml of solution A and 5 ml of solution B to obtain the colour reagent.Store in a tight container, and use within 7 days, but discard any reagent in which a colour develops.

6.0 Preparation of the assay sample

6.1 General

6.1.1 The techique 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 vitamin to be examined is prevented.

6.1.2 Fats should be melted under constant stirring. Samples shall contain portions of the surfaces as well as of the interior.

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

6.1.4 Powder and liquids shall be mixed thoroughly until homogeneity is achieved. Dry materials, such as bread, biscuits, and grains, shall be ground and mixed thoroughly.

6.2 Sample preparation

6.2.1 Accurately weigh or measure a portion of the sample to be assayed, equivalent to not less than 125 micrograms, but preferablyabout 250 micrograms of ergocalicerol. if little or no vitamin A is present in the sample, add about 1.5 mg (the equivalent of 3000 USP units) of vitamin A acetate to provide the needed pilot bands in subsequent chromatography.

NOTE-USP Means United State Pharmacopoeia.

6.2.2 Add 2 ml of cotton seed oil, then add a volume of potassium hydroxide solution representing 2.5 ml for each g of the total weight of the sample plus cotton seed oil, but not less than 15 ml, then add 50 ml of alcohol. Reflux vigoriously on a steam bath for 20 minutes, or for 30 minutes for samples weighing more than 5 g. If oil globules are evident indicating incomplete saponification, add 5 ml of potasium hydroxide solution(but not excceding a total of 100 ml), and heat again. Cool and transfer the saponified mixture to a 500 ml separator rinsing the saponification flask with a total of 50 ml of water and add each rinse to the separator. Add about 4.5 g of sodium sulphate decahydrate and 150 ml of solvent hexane. Shake vigorously for 2 minutes. When the aqueous layer has separated, transfer it to a second separator.

6.2.3 Extract with two 50 ml portions of solvent hexane, combine the extracts, and discard the aqueous solution.

6.2.4 Wash the combined solvent hexane extracts with 50 ml portions of water until the last portion shows no pink colour on the addition of phenolpthalein indicator solution. Allow the washed extracts to stand for 5 minutes, discard any water that separates, and transfer the extract to 300 ml tall form beaker containing about 5 g of unhydrous solution sulphate. Stir for 2 minutes, and decant the solution into 500 ml tall form beaker. Rinse the sodium sulphate with four 25 ml portions of solvent hexane combining the rinsings with original extracts. Reduce the total volume to about 30 ml by evaporation on a steam bath, and transfer the concentrate to a small round-bottom evaporation flask. Rinse the beaker with four 5 ml portions of solvent hexane, adding the rinsings to the flask. With the aid of vacuum in water bath at a temperature not exceeding 40^{o}C, or with a stream of nitrogen at room temperature, remove the remaining solvent completely. Dissolve the residue in a small amount of solvent hexane, transfer to volume to obtain the sample preparation.

7 Procedure

7.1 Solution of vitamin shall be protected from oxygen and actinic light.

7.2 First column chromatography

Just as the 2 ml of iso-octane disappears into the surface of the prepared first column, pipette 2 ml of the sample preparation on to the column.As the meniscus of the sample preparation reaches the column surfaces, add the first three 2 ml portions of solvent hexane, adding each succeeding portion as the preceding portion disappears into the column. Continue adding solvent hexane in portions of 5 ml to 10 ml until 100 ml has been added.

If neccessary, adjust the flow rate to between 3 ml and 6 ml per minute by application of gentle pressure at the top of the chromatographic tube.

Discard the first 20 ml of effluent and collect the remainder. Examine the column under ultraviolet light at intervals during the chromatography, and stop the flow when the front of the fluorescent and representing vitamin A is about 5 mm from the bottom of the column. (The ultraviolet lamp should provide weak radiation in the 300 nm region). It is frequently necessary to use a narrow aperture or screen with commercial lamps to reduce the amount of the radiation to the minimum required to visualized the vitamin A on the column.Transfer the elute to a suitable evaporation flask, and remove the solvent hexane completely under vacuum at temperature not above 40^{O}C or with a stream of nitrogen at a room temperature. Dissolve the residue in about 10 ml of solvent hexane.

7.3 Second column chromatography

Add the solvent hexane solution obtained as directed under first column chromatography on the second column. Rinse the evaporation flask with a total of 10 ml of solvent hexane in small portions, adding each portion to the second column and allowing it to flow through the column, and discard the effluent.When about 1 ml of the solvent hexane remains above the surface of the column, add 75 ml of the solvent and elute with the aid of gentle sunction(about 125 mmHg), collecting the elute. Evaporate the hexane under vacuum at a temperature not above 40^{o}C or with a stream of nitrogen at room temperature.

7.4 Assay preparation

Dissolve the residue obtained as directed under second column chromatography in a small amount of ethylene dichloride, transfer to a 10 ml volumetric flask, and make to volume with ethylene dichloride to obtain the assay preparation.

7.5 Colour development

Into each of three suitable matched colorimeter tubes of about 20 mm inside diameter, and designated 1, 2 and 3 respectively, pippette 1 ml of the assay preparation. Into tube 1, pippete 1 ml of the standards preparation,into tube 2, 1 ml of ethylene dichloride and into tube 3, 1 ml of a mixture of equal volume of acetic anhydride and ethylene dichloride. To each tube add quickly, and preferably from an automatic pippete, 5.0 ml of colour reagent and mix.45 seconds after the addition of the colour reagent, determine the absorbances of the three solutions at 500 nm with a suitable spectrophotometer, using ethylene dichloride as the blank.

Similarly, 45 seconds after making the first reading on each solution, determine the absorbances,of the solutions in tube 2 and 3 at 550 nm in similar manner. Designate the absorbances as A^1_{500}, A^2_{500}, A^3_{500}, A^2_{550}, A^3_{550}, respectively in which the superscript indicates the number of the tube and subsricpt the wavelength.

8.0 Calculation

Calculate the quantity of vitamin D in the portion of the sample taken by the following formula:

vitamin D in µg = *(Cs/C)(Au/As)*

Where

Cs=Concentration ot vitamin D in microgram per ml of the standards preparation.

C=Concentaration of sample in g in each ml of the final solution.

Au= Value of $(A^2_{500}-A^3_{500})-0.67(A^2_{550}-A^3_{550})$ detemined from the assay preparatiton,and

As=Value of $A^1_{500}- A^2_{500}$ determined on the solution from the standards preparation.

9.0 Repeatability

The difference between the results of a determination in duplicate (obtained simultaneously or in rapid succession but the same analyst) shall not exceed 5%.