Methods of Analysis of Vitamins

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***Abstract*— The main purpose of this review article is to provide the analytical methods for quantitative analysis of fat soluble and water-soluble vitamins. The methods include chromatography, UV Visible Spectroscopy, fluorometry and microbiological method. Vitamin analysis of food and biological samples has a major role in determining the animal and human nutritional requirements. It is important to know the composition of food to determine dietary intake to assess diet adequacy and improve the human nutrition worldwide. This review highlights a various analytical techniques used for analysis of vitamins in food samples.**

***Indexed Terms*— Vitamins, Analytical methods, Quantitative analysis.**

I. INTRODUCTION

Vitamins can either be water-soluble or fat-soluble. While many vitamins do not exist as distinct molecules, they do belong to related classes called vitamins. For example, vitamin E has 4 tocopherols and 4 tocotrienols. Humans contain thirteen vitamins, nine of which are water-soluble (vitamins B complex and C) and four of which are fat-soluble (vitamins A, D, E and K). Water-soluble vitamins quickly dissolve in water and are typically easily eliminated by the body. A constant intake is crucial because they are not digested quickly enough. Lipids (fats) are used in the intestine to digest fat-soluble vitamins. Vitamin A and D can build up in the body and cause hazardous hypervitaminosis. In cystic fibrosis, vitamin malnutrition due to fat-soluble malabsorption is particularly essential[1].

Water-soluble vitamins of the B vitamin group are crucial for maintaining healthy cell metabolism[2,3]. These vitamins are distinctly separate chemical molecules that live in the same diet, while sharing some common names. As a rule, vitamin B complex consist of eight dietary supplements[4,5]. Meat contains the maximum quantity of B vitamins. In small amounts, they can also be added to complete, unprocessed foods that depend on carbohydrates. In comparison to natural fuel, refined carbohydrates, such as sugar or white grain, have lower levels of vitamin B [6].

Vitamin C is an essential component for several animals, including humans. Animals use a number of vitamins to help them generate vitamin C. Numerous dietary supplements contain sodium and calcium ascorbate salts. The digestive process breaks down these releases. The body produces ascorbate and ascorbic acid, which are subsequently converted into one another by pH. Reducing agents transform dehydroascorbic acid from oxidised molecular forms to ascorbic acid. [7]

The two categories of vitamins are fat-soluble vitamins and water-soluble vitamins.

FAT SOLUBLE VITAMINS: These vitamins are fat-soluble and stored in the body for longer lengths of time than water-soluble vitamins, making them more likely to be harmful when consumed in excess.

EXAMPLE: A, D, E, and K vitamins

WATER SOLUBLE VITAMINS:
Some vitamins are water soluble. Extra water-soluble vitamins are thought to be eliminated through the urine.

EXAMPLE: Vitamin B complex with vitamin C

II. METHODS OF ANALYSIS OF VITAMINS

(1) CHROMATOGRAPHIC METHOD

Many chromatographic methods have been developed for vitamin analysis. It includes liquid chromatography techniques like high-performance liquid chromatography (HPLC), ultra-high performance liquid chromatography (UHPLC) and gas chromatography (GC).

EXAMPLE: THIAMINE AND RIBOFLAVIN (VITAMIN B1 and VITAMIN B2)

As a natural component found in many foods and also added as an essential nutrient in food items, thiamin commonly known as vitamin B1 is a water-soluble vitamin. Beriberi, neuralgia, and other conditions have been treated with it as well as prevented. Additionally, it is included in pharmaceutical dosages and foods and beverages that have been fortified with vitamin B1. For the preservation of neutral activity and the metabolism of carbohydrates, it is essential.[8]

Riboflavin is the precursor for two coenzymes: riboflavin 5-mono-phosphate (FMN) and flavin adenine dinucleotide (FMD), in addition to some covalently bound flavins, which are found in enzymes like succinate dehydrogenase. Among the several redox reactions that riboflavin participates in are the metabolism of fatty acids and the electron transport chain in the mitochondria. Deficits, which are common in third-world countries due to poor diets, lead to mucocutaneous surface sores.[9]

PRINCIPLE: This method is based on the fluorescence detection of thiamin's oxidised form i.e., thiocrome after chromatographic clean-up, extraction, and enzymatic hydrolysis of its phosphate esters.

PROCEDURE: The procedural sequence for this method are outlined below. The precolumn SepPak C-18 (waters, Division of millipore , Melford , MA) cartridge can be reused repeatedly for the concentration of these vitamins.

Weigh sample and extract including the enzyme treatment.

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Elute thiamin and riboflavin with 5 ml 50% MEOH into a 5 ml volumetric flask, then oxidise 10 ml of the diluted extract by passing it through a sepPak (Water Associates) cartridge, followed by 5 mL 0.01 M phosphate buffer, pH 7.0

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Use a 4.6 mm X 25cm ultrasphere ODS, 5 column, a 20/80 MEOH/H_20 mobile phase containing 0.005 M tetrabutylammonium phosphate, and fluorescence detection at 360 nm/415 nm ex/em to separate the vitamin using HPLC.

(2) UV VISIBLE SPECTROPHOTOMETRIC METHOD

This method is effective, sensitive, and trustworthy for finding vitamin B3 and its vitamin nicotinamide in samples of human hair. It is comprehensive for the B3 vitamin niacin test. This newly created hair test can be used in clinical examinations in addition to blood and urine tests for the long-term deficit, identification, and certification of nicotinamide.

EXAMPLE: VITAMIN-A (RETINOL)

The majority of vitamin A analysis is done using colorimetric techniques. Multiple Lewis acids and vitamin A work together to momentarily create the colour blue[10].Another method that has been accepted by the Association of Official Analytical Chemists (AOAC) is a solution of vitamin A, which was extracted from food using nonpolar anhydrous solvents, can be used to measure the amount of vitamin A in food products by reacting it with a solution of 20–25% antimony trichloride in chloroform. The created blue hue reaches its maximum intensity in 5 to 10 seconds before swiftly fading. This process is also known as the Carr-Price[11,12] technique.

PRINCIPLE: This technique examines the colour instability at A620 nm caused by the interaction of vitamin A with $SbCl_3$. There is no distinction between vitamin A and $SbCl_3$ in the colour reaction. Retinol isomers and retinyl esterts are not distinguished by the colour response. Retinaldehyde, dehydroretinol, and related chemicals are used to create colours, and their greatest absorption occurs at slightly longer wavelengths.

PROCEDURE: Before saponification, pyrogallol, an antioxidant, is added. The removal of these compounds by column chromatography or in the case of low carotenoid concentration in comparison to the

vitamin A content, readings of a chloroform extract at 440 to 460 nm without prior chromatographic separation are two ways to correct for the presence of carotenoids. If there are no carotenoids in the sample being examined, these remedial actions are not necessary.

Measure out the proper amount of the sample, then homogenise.
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For 30 minutes, saponify with ethanolic KOH (add antioxidant).
+
Move to a different funnel, add water, then extract with a volume of hexane between one and five, repeat the extraction process, and combine the extracts. Repeatedly rinse the extract with the same amount of water
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To produce up to volume, filter 5 g of anhydrous Na_2SO_4 through paper into a volumetric flask, then rinse the filter with hexane.
+
To produce up to volume, filter 5 g of anhydrous Na_2SO_4 through paper into a volumetric flask, then rinse the filter with hexane.
+
Create a standard curve by creating standard dilutions with A620 values ranging from 0.07 to 0.7 and plotting them against g of vitamin A using the USP vitamin A reference standard.
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At 620 nm, calculate the sample's vitamin A concentration as follows: Hexane is removed from the standard solution in the colorimetry tube by evaporation. Then, 1 mL of chloroform and a measured amount of $SbCL_3$ Solution are added. The colorimeter is then set to 0 Abs (or 100%). Remove the hexane from the sample and standard solutions, then add 1 mL of chloroform and the same quantity of $SbCl_3$ solution that was added to the standard blank mentioned above. Read the results right away.

EXAMPLE (2) : VITAMIN C (ASCORBIC ACID)
Assessments of vitamin C status have used plasma or serum, buffy coat or pure white cells, and cold blood or urine excretion following a test dose[13]. At -70°C, or -196°C, Vitamin C can be stored without adding acid for 3 weeks even though it quickly degrades at neutral pH at higher temperatures[14].

PRINCIPLE: The indicator dye causes L-ascorbic acid to oxidise into L-dehydroascorbic acid. Excess unreduced dye is rose-pink in acid solution at the process' conclusion. By first reducing it to L-ascorbic acid with an appropriate reducing agent, L-dehydroascorbic acid can then be identified.

PROCEDURE: It is advised to use an EDTA (ethylene diamine tetraacetic acid) chelating agent with the extraction when the biological matrix to be examined contains considerable levels of iron or copper.

The red-colored endpoint must be valid for at least 10 seconds. The endpoint is inaccessible to visual inspection with coloured samples like red beets or goods that have been severely browned. As a result, in these situations, it must be determined by using a spectrophotometer with a 545 nm wavelength setting to see the change in transmittance.

Weigh the sample and extract it by homogenising it in a solution of metaphosphoric acid and acetic acid (15g HPO_4 and 40mL HOAc in 500 mL H_2O).
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Prepare a standard response: 50 mg of L-ascorbic acid in 100 mL of water
+
Sample extract should be filtered (and/or centrifuged) before being properly diluted to a final concentration of 10-100 mg ascorbic acid per 100 mL.
+
Titrate three replicates of the standard and the sample, separately, with dichloroindophenol solution, until a pink endpoint is reached that lasts for at least 10 seconds.

3) FLUOROMETRIC METHOD
The fluorescence produced by some vitamins when they interact with a fluorophore is directly proportional to the vitamin concentration, which is the basis for the flurometric approach.

EXAMPLE : THIAMINE (VITAMIN B1)

Aside from a few infrequently used functional test, there are three different ways to assess thiamin levels. These includes:

- The test for excretion of thiamin in the urine. [15],[16]
- The detection of thiamin concentrations in whole blood ,EDTA, or heparin-infused plasma, as well as the measurement of erythrocyte transketolase and its reactivation in vitro by the addition of the cofactor thiamin pyrophosphate (cocarboxylase).[17],[18]and
- The measurement of thiamin levels in plasma, whole blood, EDTA, or heparin.[17],[19]

PRINCIPLE: This method is based on the fluorescence measurement of thiamin's oxidised form, thiochrome, after extraction, enzymatic hydrolysis of the phosphate esters, and chromatographic cleanup.

PROCEDURE: With some matrices, such as vitamin concentrations that contain nonphosphorylated thiamine, the enzymatic treatment or the chromatographic clean-up may not be essential.

Weigh enough of the composite and finely ground sample so that it contains approximately 10 to 20g of thiamine, then add 0.14 N HCL, mix, and autoclave for 15 minutes at 121° C before cooling.

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pH should be adjusted to 4.5—5.0 with HCL, followed by the addition of 5mL of 6% enzyme solution (Mylase 100, Miles Laboratories, or takediastase, pfalz and bauer), and incubation for 3 hours at 45—500°C. Once cool, the pH should be adjusted to 3.5, followed by dilution to volume with water, mixing, and filtering.

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Subject standard solution to the same enzyme treatment, but separate from sample.

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Purify by gravity-flowing 5—25 mL of sample extract through a 6—8 mm X 15 cm column filled with 50—100 mesh Bio-Rex 70 (BioRad Laboratories). Elute thiamin with 5 mL parts of hot acid KCL in a 25 mL volumetric flask after washing with 3 x 10 ml of hot water. Respect all standards equally.

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Convert thiamin to thiochrome as follows in dim light: Add 5 mL of sample or standard eluate to each of the four test tubes, each of which contains 1.5 g of sodium chloride. Add 15 mL of isobutyl alcohol right away to two tubes after quickly adding 3 mL of $K_3Fe(CN)_6$ Solution. Shake vigorously for 90 seconds, centrifuge, and decant the isobutyl alcohol fraction into a fluorescence reading tube. Same care should be taken with the remaining two tubes, but instead of the ferricyanide solution, add 3mL of 15% NaOH. Give standard solution the same treatment.

4) MICROBIOLOGICAL METHOD:

Certain microorganisms, such as Lactobacillus casei and Lactobacillus plantoides, whose growth depends on specific vitamins, are quantified using microbiological methods. Often, a negative control is employed that contains zero vitamin. The assays used in microbiology are very sensitive and precise. They are frequently used to examine water-soluble vitamins.

EXAMPLE (1): VITAMIN-D (CALCIFEROL)

The biochemical measurement of vitamin D status relies on the measurement of 25(OH) vitamin D levels in serum or plasma, because this derivatives, of all the forms of vitamin D present, reflects most accurately whole body supplies of vitamin D. There are considerable seasonal variations in serum levels of (OH)-D in people living in temperature countries, reflecting variations in sunlight exposure.[20]

PRINCIPLE: Microorganisms ability to grow is directly correlated with how much of a certain vitamin they require. As a result, in microbiological tests, the development of a specific microbe in an extract of a substance that includes a vitamin is compared to the development of that microbe in the presence of known concentrations of that vitamin. Bacteria, yeast, or protozoan organisms could be utilised as test organisms. Growth can be measured by gravimetry, acid production, or turbidity. The technique most usually used to measure bacteria and yeast is turbidimetry. Clean sample and standard extracts are also a quicker approach than turbidity measurements if turbidity measurements.

Sample extraction

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include in the diet of rats

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Utilize rats fed a diet low in vitamin D for 19-25 days.

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Rats were given known and unknowledgeable doses of vitamin D.

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rat-killed bone calcification measurement: Put a particular bone in 1.5% $AgNO_3$: Get AG_3PO_4 ppt; Ag+ Complexes with PO_4 in calcified locations; dark spots (lines) are calcified regions; assign score depending on line darkness. compare the unknown with the standard curve.

EXAMPLE (2): NIACIN

In the microbiological assay of Niacin, Lactobacillus plantarum is the test organism. Turbidity is generally used to quantify growth. Acidimetric measures can also be employed when lactobacilli are the test organism.

PROCEDURE : The freeze-dried culture needs to be inoculated on bacto-lactobacilli agar, incubated at 370ºC for 24 hours, and then stored under refrigeration until use, but no longer than 2 to 4 weeks prior to retransfer or inoculum production. This is how a stock culture is prepared and maintained.

Prior to sample and standard inoculation, the inoculum is prepared by transferring the stock culture to a tube containing bacto-lactobacillus broth and culturing it at 370C for 24 hours. If the inoculum culture does not grow well, a second transfer may be necessary.

Turbidity is generally used to quantify growth. Acidimetric measures can also be employed when lactobacilli are the test organism. The latter may be required if a clear sample extract, which is required for turbidimetry, cannot be obtained before inoculation and incubation.

INOCULUM : Prepare using Bacto-Lactobacilli broth AOAC and L.plantarum, ATCC 8014 culture.

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SAMPLE PREPARATION: Weigh out enough sample to ensure that it contains ca.0.1 mg of niacin, add 1N H_2SO_4, macerate, and autoclave for one hour at 121°C before cooling. Niacin should be diluted to about 0.1 g per millilitre, adjusted to pH 6.8, combined, and filtered.

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ASSAY TUBE PREPARATION: Use 0.0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mL of sample filtrate in at least two separate tubes. Fill the remaining space to 5.0 mL with water. Add 5.0 mL of Difco Basal Medium for Niacin Assay broth to each tube. Autoclave for 10 min at 121° C. And let cool.

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STANDARD PREPARATION: Make at least two assay tubes using 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 mL of standard solution (0.1 l/mL niacin), fill the remaining space with water, add 5.0 mL of assay broth, and treat the two tubes the same as sample tubes.

INOCULATION AND INCUBATION: One drop of inoculum should be added to each tube. The tubes should then be covered and incubated at 370°C for 16—18 hours, or until the highest level of turbidity is noticed in the tubes with the highest concentration of niacin.

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DETERMINATION: At any wavelength between 540 and 660 nm, measure %T.

CONCLUSION

Several analytical methods like chromatography, spectroscopy, fluorimetry and microbiological methods of assay has been mentioned as it essential for the quantification of vitamins in food sample.

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