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Different Methods Used For Determination of Vitamin C: A Review

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A B S T R A C T

Vitamin C or ascorbic acid is essential for human life and is required for a range of physiological functions in the human body. It is naturally present in fresh fruits and vegetables. Ascorbic Acid (AA) is a natural and powerful water-soluble antioxidant associated with long-lasting food products. It has been widely used in the pharmaceutical, chemical, cosmetic and food industry as an antioxidant. Therefore, there is a need to find an accurate and reliable method for measuring the amount of ascorbic acid in a sample. The purpose of this review is to discuss different methods used for determination of vitamin C. The different methods used for the determination of vitamin C are UV-spectrophotometry, high-performance liquid chromatography (HPLC), dye titration method, iodometric titration, iodimetric titration, enzymatic methods, fluorometry and voltammetry.

Introduction

Vitamins are an integral part of our diet and play an important role in human health. The specific coenzymes involved in metabolism and other specialized activities are essentially composed of these vitamins. The essential micronutrient required for normal metabolic function of the body is vitamin C (ascorbic acid). Due to mutation in the gene coding for the enzyme L-gulonolactoneoxidase, humans and other primates, guinea pigs and fruit-eating bats have lost the ability to synthesize vitamin C. The enzyme, L-gulonolactone oxidase, is essential for the biosynthesis of vitamin C via the glucuronic acid pathway (Mussa and Sharaa, 2014; Woodall and Ames, 1997). Thus, there is no way to obtain vitamin C other than through the diet. Important role is being played by Vitamin C as a component of enzymes involved in the synthesis of collagens and carnitine and it is the major water – soluble antioxidant in the body (Sies *et al.*, 1995; Levine, 1986; Levine *et al.*, 1995). Vitamin C lowers blood pressure and cholesterol level (Rath, 1993) and markedly reduces the severity of a cold and prevents secondary viral or bacterial complications. For maintaining good and sound health and for prevention from common cold, the human body should be kept saturated with vitamin C (Nelson and Cox, 2004). The risk of developing breast cancer, cervix, colon, rectum, lung, mouth,

prostate and stomach cancer can be effectively lowered by vitamin C (Levine *et al.,* 1996; Block, 1992; Block, 1991; Feri, 1994; Jacobs, 1993). Vitamin C has the potential for use as an epigenetic regulator, immunotherapy enhancer and is known to show anti-cancer functions (Mussa *et al.,* 2022). Vitamin C has several pivotal physiological functions in the body. It is a powerful antioxidant and protects oxidation of macromolecules like proteins, fats, and DNA (Mussa *et al.,* 2022). This vitamin is especially plentiful in fresh fruit and fruit juices, in particular citrus fruits, and vegetables (Bendich, 1997). Vitamin C was first isolated in 1928 and in 1932 that it was identified as the agent which causes scurvy. Scurvy is a condition which occurs due to deficiency of vitamin C in the body (Levine, 1986). Scurvy can be prevented with the intake of as little as 10 mg vitamin C per day (Weber *et al.,* 1996). It participates in numerous biochemical reactions like bone formation to scar tissue repair (Groff *et al.,* 1995). Ascorbic acid (AA)is a six-carbon sugar containing a diol group at carbons 2 and 3 and which is readily oxidized to a diketo group to form dehydroascorbic acid (DHAA) (Tee *et al.,* 1988). Its readily oxidation to dehydroascorbic acid is the most prominent chemical property of the vitamin.

The recommended daily intake of vitamin C varies according to age, sex, risk group (cigarette smokers, alcohol users, institutionalized elderly and subjects on certain drugs) and criteria applied in individual countries. The recommended dietary allowances (RDA) for vitamin C in India are 80 mg/day for men and 65 mg/day for women based on ICMR-NIN, 2020 data. The RDA for all other groups according to Indian standards are listed in the Table I.

The recommended dietary allowances for vitamin C increases in case of smokers, pregnant women and lactating mothers (Silva *et al.,* 1999). The RDAs are in a similar range in other countries. The current recommended daily acceptance (RDA) for ascorbic acid is suggested to be more than existing levels to reduce the risk of heart diseases, stroke and cancer in healthy individuals (Tee *et al.,* 1988).

Vitamin C is sensitive to degradation during food processing and storage, and that is why it is used as an index for nutrient quality in different food products (Pathy, 2018; Ozkan *et al.,* 2004). Consequently, studies on vitamin C content in foods are important for nutritional labels, food databases and for establishment of dietary reference intakes. During storage, vitamin C of fruit juices is readily oxidized (Kabasakalis *et al.,* 2000). The oxidation of L-ascorbic acid (L-AA) occurs rapidly to Dehydroascorbic acid (DHAA) and the factors affecting the oxidation include pH, high temperatures, light, presence of oxygen, enzymatic action and metal ions like Fe^{3+}, Ag^{+}, Cu^{2+}. The factors influencing the degradation of vitamin C in packaged fruit juices include storage temperature, pH, dissolved oxygen, residual hydrogen peroxide and trace metal ions (Ozkan *et al.,* 2004). As DHAA has equivalent biological activity, it is important to measure both DHAA and L-AA to know the vitamin C content of different food stuffs. In fresh horticultural products the amount of DHAA is less than 10%, but this tends to increase during storage. The analytical method used for determination of vitamin C should measure the actual content without shifting the equilibrium of the two molecules. So any degradation of L-AA should be avoided during extraction and analysis of vitamin C. It is known that irreversible hydrolysis of DHAA will produce 2,3-diketo-L-gulonic acid which is biologically inactive, followed by other products like oxalic acid, L-Xylose, L-Threonic acid, L-Xylonic acid and CO_2 etc. DHAA can be converted back to L-AA by using reducing agents like glutathione dehydrogenase (in vivo), homocysteine and dimercapto propanol (in vitro) (Castilho *et al.,* 2014). Vitamin C degradation can occur by anaerobic and aerobic pathways. The aerobic degradation may occur during processing of food while the anaerobic degradation might occur during storage. Hydroxymethylfurfural (HMF), one of the decomposition products of vitamin C degradation is also supposed to be a precursor of brown pigments (Karadeniz *et al.,* 2005). It therefore becomes important to carefully select the method for vitamin C determination as storage, sample preparation and extraction conditions could

affect the degradation of L-AA. Vitamin C is used as a food additive in the food industry, where it preserves and protects food from any colour changes in addition to acting as a nutrient (Karadeniz *et al.,* 2005). It is added to foods as a nutrient to compensate for processing losses, as an antioxidant (El Ishaq *et al.,* 2015; Sheree *et al.,* 2016) and as an agent to prevent the browning of fresh/ canned fruits and vegetables (Zhang *et al.,* 2022; Kidon *et al.,* 2023). It also aids in iron absorption and collagen formation (Lis *et al.,* 2022). Vitamin C is added to many fruit juices, fruit-flavoured beverages, juice-infused sodas, smoothies, cereal-based products, and milk (Kowalska *et al.,* 2023). Keeping in view its importance; the estimation of vitamin C assumes significance. Nutritional value of fruits and vegetables and the estimation of vitamin C content is very important for the better utilization of the fruits and vegetables as human food (Mussa and Sharaa, 2014; Khan *et al.,* 2005). The development of rapid, simple, and inexpensive analytical methods for determination of vitamin C is one of areas of growing interest, as quick decisions are needed in food and pharmaceutical industry. Many analytical methods are used for vitamin C determination, including titrimetric, spectrophotometry, chromatography, voltammetry and fluorometry etc. Although there are many available methods, not all methods can be employed for determination of both the forms of vitamin C i.e. ascorbic acid and its oxidized form, dehydroascorbic acid. The two forms have different chemical, optical and electrochemical properties.

Methods used for Determination of Vitamin C

Spectrophotometric method

UV Spectrophotometry is mostly used to determine ascorbic acid as it can absorb UV rays and is a simple method. This method can be used for vitamin C tablets, fresh or packaged fruit juices, solid fruits and vegetables (Desai and Desai, 2019). To determine the content of total vitamin C, a well - established method is the 2, 4-dinitrophenyl hydrazine (2, 4-DNPH) method (Mohammed *et al.,* 2009). There is a coupling reaction between 2,4 DNPH and ascorbic acid. This ascorbic acid content in different fruits and vegetables can be determined by using this method. In this method the total amount of vitamin C includes both Ascorbic acid and Dehydroascorbic acid. In this method, bromine water in the presence of acetic acid oxidizes the ascorbic acid into dehydroascorbic. Then a known amount of 2, 4 DNPH is added which gives a coupling reaction. Solutions are kept for 3 hours. After 3 hours 85% H2SO4 is added which gives a colored solution. The absorbance of these solutions is measured using UV spectrophotometer and the ascorbic acid content is then determined (Desai and Desai, 2019).

A second method involves determination of vitamin C spectrophotometrically by reduction of the absorbance of a potassium permanganate solution when reacted with a vitamin C solution in acidic medium, as shown in the equation below. In this reaction, the vitamin C is oxidized and the potassium permanganate (violet coloration) is reduced causing a decrease in the absorbance at 525 nm. This method is based on a previous study (Lenghor *et al.,* 2002).

$2KMnO_4 + 5C_6H_8O_6 + 3H_2SO_4$ ------> $2MnSO_4 + 5C_6H_6O_6 + K_2SO_4 + 8H_2O$

Dye titration method or Dichlorophenolindophenol (DCPIP) method

This method (AOAC method, 2012) makes use of the reducing power of the vitamin, and employs 2, 6- dichlorophenolindophenol (DCIP) as the redox indicator for the determination of ascorbic acid. The principle of this method is a titration of ascorbic acid with dichlorophenolindophenol.

Ascorbic acid reacts with DCPIP, changing the color from blue to colorless. The food samples are extracted using metaphosphoric acid (MPA) and the pH adjusted to 1.2. The extract is then titrated against 2,6-dichlorophenol indophenol. In this titration which is a redox reaction, ascorbic acid in

the extract is oxidized to Dehydroascorbic acid (DHAA) and the dye is reduced to a colourless compound. The end point of this titration is a pink colour due to the excess unreduced dye in acid solution. In case of intensely colored extracts repeated ether extraction is carried out to facilitate easy detection of end point (Tee, 1988).

They react in a 1:1 fashion, so if a known quantity of DCPIP solution reacts with the plant tissue extract, the quantity of DCPIP used gives a direct measure of the quantity of ascorbic acid present (Patrick *et al.*, 2016).

DCPIP titration is suitable for fresh juices and multivitamin supplements that do not contain significant quantities of copper or iron. However, highly colored extracts from fruits and vegetables, for example, can mask color changes at the end point (Daud *et al.*, 2016).

Iodometric method

Vitamin C ($C_6H_8O_6$) can also be determined by iodometric titration. There are several oxidizing agents such as potassium iodate, potassium permanganate and potassium dichromate which have higher reduction potential values than iodine.

Higher the reduction potential value, stronger is the oxidizing agent. Thus potassium iodate, potassium permanganate and potassium dichromate are stronger oxidizing agents than iodine (I_2).

Oxidising agent + KI ------> I_2 + reduced form of oxidizing agent

In this titration, potassium iodate is added to a solution of ascorbic acid which contains a strong acid like sulphuric acid and potassium iodide (KI). The potassium iodate on reacting with KI, liberates I_2. The iodine produced reacts with ascorbic acid leading to the formation of dehydroascorbic acid ($C_6H_6O_6$) and iodide ions (I^-).

$KIO_3 + 5KI + 6H^+$ ------> $3I_2 + 6K^+ + 3H_2O$

$C_6HO_6 + I_2$ ------> $C_6H_6O_6 + 2I^- + 2H^+$

The potassium iodide is always added in excess to ensure completion of reaction and to dissolve the iodine. After complete utilization of ascorbic acid, the excess I_2 remaining in the solution can be titrated with sodium thiosulphate solution using starch as an indicator. Starch forms an intense blue colored complex with I_2 and at the end point of the titration the blue colour disappears.

$I_2 + 2Na_2S_2O_3NaI + Na_2S_4O_6$

By the amount of sodium thiosulphate used in the titration the remaining I_2 after reaction with ascorbic acid can be calculated. Thus iodometry is an indirect titration for estimating the concentration of ascorbic acid (Dioha *et al.*, 2011).

Iodimetric method

The conversion of I_2 to iodide (I^-) is a reversible reaction. The reduction potential of this system is around 0.54 V. There are many substances which have a lower reduction potential than iodine and such substances have a tendency to lose electrons to I_2 and act as reducing agents.

Such titrations where iodine is used to determine the concentration of a reducing agent are known as iodimetric titrations. One such reducing agent is ascorbic acid and in this method the ascorbic acid solution is titrated with an iodine solution using starch as an indicator. In this titration I_2 is reduced to I^- and ascorbic acid is oxidized to dehydroascorbic acid.

Ascorbic acid + Iodine (I_2) --------> 2 I^- + Dehydroascorbic acid

When the ascorbic acid is completely oxidized to dehydroascorbic acid, the excess iodine reacts with the starch indicator and forms a blue-black starch-iodine complex. This is the endpoint of the redox titration. The iodimetric method is simpler than the potassium iodate method (Satpathy *et al.*, 2021).

High Performance Liquid Chromatography (HPLC) method

The high-performance liquid chromatography (HPLC) technique deserves an increasing interest mainly due to its rapidity, selectivity, specificity properties and simple sample preparation procedure (Mohammed, 2009). The sample preparation and sample extraction are important steps to minimize degradation of L-AA. Metaphosphoric acid (MPA) is the most common reagent used as it can function both as an extractant and stabilizer.

It inhibits ascorbate oxidase, metals catalysis, and precipitates proteins. MPA can be used with other organic acids and modifiers like acetic acid, methanol etc and stabilizers like ethylenediamine acetic acid (EDTA) and monosodium glutamate (MSG). L-AA has higher stability in MPA than oxalic acid and trichloroacetic acid. The analysis of liquid samples is done by directly injecting the sample in HPLC after dilution with initial mobile phase.

The solid samples are first homogenized with the extractant, followed by centrifugation or filtration and then ingected in HPLC after dilution with mobile phase. The reversed phase-HPLC is the most common approach used because of the non-volatile and hydrophilic nature of vitamin C. Other methods like ion-exclusion, ion-exchange, ion-pair and hydrophilic liquid chromatography have been used.

Ultra high performance liquid chromatography (UHPLC) has also been used in analysis of vitamin C due to shorter time of analysis and lower solvent consumption when compared with other approaches. L-AA shows a strong absorption in the UV region (245-270 nm) and this makes UV absorbance the most popular detection techniques. Other detection techniques like mass spectrometry, fluorescence and electrochemical detection can also be used (Castilho *et al.*, 2014).

The HPLC method is a procedure used extensively in research and in the food industry due to its high accuracy and the ability to measure both AA and the oxidized form of vitamin C, dehydroascorbic acid (DHA).

Micro fluorometric method

In the micro fluorometric method (also as given in AOAC, 1984), the food samples are first extracted intometaphosphoric acid solutions. Ascorbic acid in the extracts is oxidized to dehydroascorbic acid with Norit (active carbon). The aliquots are then reacted with o-phenylenediamine to give fluorescent quinoxaline derivative. The quinoxaline derivative on activation at 350 nm, fluoresces at 430 nm. For each food sample analyzed, a specific sample blank was carried out by the addition of boric acid (Tee, 1988).

Enzymatic method

The enzyme ascorbate oxidase catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. The dehydroascorbic acid formed as a result is reacted with *o*-phenylenediamine to produce a 2, 2-anhydroquinoxaline. The absorption of 2, 2-anhydroquinoxaline with respect to a specially prepared blank is observed at 358 nm using a UV spectrophotometer. The increase in the absorbance of the quinoxaline is an indicator of the amount of ascorbic acid present and that has reacted with oxygen in the presence of ascorbate oxidase.

Voltammetric method

Among the different analytical methods used for determination of trace concentrations of ascorbic acid, voltammetric techniques have been considered as important. Electrochemical methods are promising alternatives, because of their quick response times, low cost, simplicity of instrumentation, high sensitivity, and also they are environmentally friendly (Desai and Desai, 2019; Patrick *et al.*, 2016; Dioha *et al.*, 2016). In these techniques by applying variable potential, current arising from oxidation or reduction on an electrode is measured (Smyth, 1992).

Table.1 RDA of Vitamin C for Indians -ICMR-NIN, 2020

S. No.	Group	Category of work and age	Body weight (kg)	Vitamin C (mg/d)
1	Men	Sedentary	65	80
		Moderate		
		Heavy		
2	Women	Sedentary	55	65
		Moderate		
		Heavy		
		Pregnant woman	55+10	80
		Lactation		130
3	Infants	0-6m	5.8	20
		6-12m	8.5	30
4	Children	1-3y	12.9	30
		4-6y	18.3	35
		7-9y	25.3	45
5	Boys	10-12y	34.9	55
		13-15y	50.5	70
		16-18y	64.4	85
6	Girls	10-12y	36.4	50
		13-15y	43	65
		16-18y	46	70

Fig.1 Structures of L-Ascorbic acid (L-AA) and Dehydroascorbic acid (DHAA)

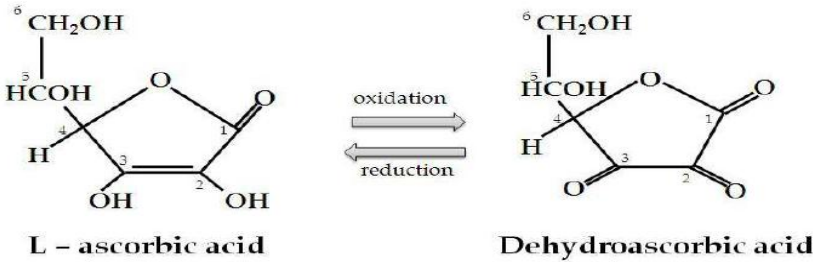


Fig.2 Reaction of Dehydroascorbic acid with 2,4-Dinitrophenylhydrazine

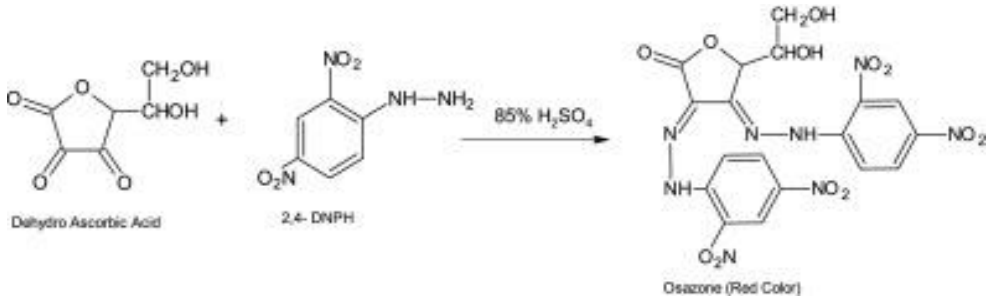
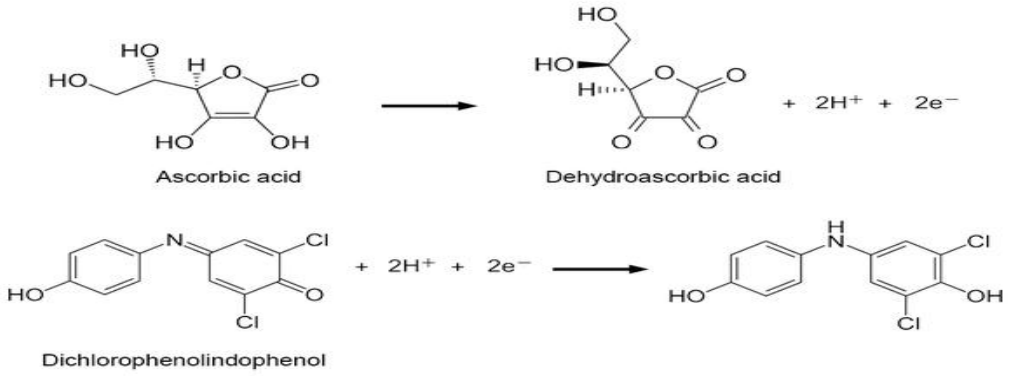


Fig.3 Reaction of Ascorbic acid with Dichlorophenol indophenol



This is commonly done with an instrument called a potentiostat, which for these measurements is capable of applying variable potentials to the working electrode relative to a reference electrode (like Ag/AgCl) while measuring the current that flows as a result of the electrode reaction. The result from the experiment comes in the form of a voltammogram, which is the plot of the current versus the potential of the working electrode. Depending on the particular method, it is possible to apply reducing and/or oxidizing potentials. Different voltammetric methods include square wave voltammetry (Osteryoung, 1986), linear sweep voltammetry, cyclic voltammetry, differential pulse voltammetry (Bond, 1980),adsorptive stripping voltammetry (Osteryoung and Schreiner, 1988), anodic or cathodic stripping voltammetry (Oveisi *et al.*, 2002; Jannat *et al.*, 2009) and electrochemical immunoassay (Wang, 2021). A number of voltammetric electrodes or cells are introduced and are used in industry and research. These electrodes are referred to as sensors, which are used for the analysis of various types of analytes in different media. Different attempts have been made to develop simple and rapid electrochemical methods for determination of ascorbic acid in different samples (Satpathy *et al.*, 2021; Ahmida, 2009; Esteban and Ho-Chu, 1997). Modifying the surface of the working electrode is of great importance since it improves electrochemical performance by improving sensitivity, electron conductivity, and surface area as well as mechanical properties (Mohamed, 2009; Esteban and Ho Chu, 1997). Cellulose acetate film modified glassy carbon electrode (Mussa and Sharaa, 2014), Pt electrode (Woodall and Ames, 1997), MWCNT/ tetradecyltrimethylammonium bromide modified glassy carbon electrode (Desai and Desai, 2019), AgNPs/PVP modified glassy carbon electrode (Ahmida, 2009), and multiwall carbon nanotubes modified glassy carbon electrode (Esteban and Ho Chu, 1997) are among commonly reported electrode materials for the electrochemical determination of ascorbic acid in pharmaceutical formulations and fruit juices.

Iodine-coated platinum electrode is another remarkable electrode due to its application, sensitivity, simplicity in preparation and environment friendliness. It is one of the most suitable and simple method for the determination of ascorbic acid in pharmaceutical products (Mohammad *et al.*, 2021). For the estimation of ascorbic acid using voltammetric techniques, suitable voltammetric technique and the working electrode is chosen depending upon the sample. Electrolyte and the standard solution of ascorbic acid are prepared using high quality chemicals and reagents. Samples are prepared and voltammograms are recorded and the ascorbic acid is estimated in the given samples.

The oxidation-reduction titration method or the DCPIP method is the most commonly used method for determination of vitamin C. In this method ascorbic acid is oxidized to dehydroascorbic acid

and the indophenol dye is reduced to a colorless compound. It is a simple and easy method to determine vitamin C in fruits but the method is not suitable for fruits that have reddish-purplish color. The titration method also is time-consuming and lacks specificity due to interference of reducing substances in the food such as ferrous iron, stannous tin, cuprous copper and sulphur dioxide etc. This method can only be used in cases when the concentration of dehydroascorbic acid is negligible as only ascorbic acid content can be determined by the DCPIP method. However, the sample preparation does not require the complicated procedure needed for other methods.

The advantage of the DCPIP method over the iodine and iodometrictitration method is that fewer chemicals are used and as a result is less time consuming. The method based on titration with iodine solution is more convenient as compared to the iodometric titration which involves many reagents and is time consuming. All the above titrations can only determine the amount of ascorbic acid and not dehydroascorbic acid.

The enzymatic method used for analysis is highly specific, requires low temperatures and as compared to chemical methods uses less hazardous reagents. UV Spectrophotometry is a simple method and is used for determination of Vitamin C as it is able to absorb UV rays. Spectrophotometric method for determination of vitamin C is a simple and reliable method (Desai and Desai, 2019). The DNP method used in spectroscopy has an advantage that it determines both ascorbic acid and dehydroascorbic acid. The DNP method used often produces errors, as only 85% of dehydroascorbic acid reacts with DNP at 37ºC for a period of 3 hours, and slight fluctuations in the incubation temperature and time affect the data (Tsumura *et al.,* 1993). But inspite of this the assay has the advantage of being simple and this makes the technique widely accessible and reproducible when compared with the titrimetric method. One of the major concerns in using the spectrophotometric procedures for estimation of vitamin C in foods is the use of several chemicals, like bromine which must be handled with great caution. This method is of less use for highly colored solutions, unless proper measures are taken (Freed, 1966). Although the UV method is less sensitive than HPLC procedure, it is easy to use in conventional research laboratories.

HPLC method is simple, rapid, reproducible and highly specific to ascorbic acid in the presence of a variety of excipients and this demonstrates that this method would be particularly suitable for the determination of ascorbic acid. The HPLC method can be used to determine both the forms of vitamin C (ascorbic acid and dehydroascorbic acid) and also other water soluble vitamins simultaneously. However, the cost of the equipment is high.

The voltammetric techniques to estimate ascorbic acid have many advantages over other techniques. They are cost effective, simple instrumentation, environment friendly and highly sensitive. Moreover, the working electrodes can be modified according to the sample used.

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