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Spectrophotometric and titrimetric analysis of phytoascorbate

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Abstract

Two phytoascorbate (vitamin C) quantification protocols viz., spectrophotometric (DNPH) and titrimetric (Iodine and DCIP), were comparatively evaluated for the analytical efficiencies using 16 different botanicals. Results were validated by performing a relative phytoascorbate recovery study. Spectrophotometric quantification process (DNPH) showed highest quantum of ascorbate in all the botanicals as compared to that of the titrimetric (Iodine and DCIP) process. The quantum of ascorbate measured by DCIP and Iodine method was almost at parity with each other. The highest value of vitamin C mg /g dry weight (28.340) was recorded in *E. officinalis* fruit pulp followed by *Moringa oleifera* leaves (20.437), *Carica papaya* leaves (11.987) *Citrus lemon* peel (11.765) and *Citrus lemon* leaves (10.870).

Keywords: Ascorbic acid, vitamin C, quantification, plants....

Introduction

Vitamin C, commonly known as ascorbic acid or ascorbate (abbreviated as VC here) is a multifunctional compound widely distributed in nature, particularly in the plant kingdom. It occurs in relatively high concentrations in fruits and vegetables but to a much lesser extent in animal tissues and animal-derived products. Owing to the great importance of VC, its quantitative analysis has gained increased significance in several areas of analytical chemistry such as pharmaceutical and food applications. It is also clinically important to determine its concentration in blood, urine and some tissues. Many methods depend on the reaction of VC by means of suitable reagents, then the product of reaction or excess reagent monitored optically (spectrophotometric, Guelu *et al.*, 2005 and fluorimetric, Perez-Ruiz *et al.*, 2004) [8, 17], electrochemically (amperometric, Fei *et al.*, 2004; voltammetric, Dursun and Nisli, 2004 or potentiometric, Nazer *et al.*, 2004) [7, 5, 15] and chromatographically (High Pressure Liquid Chromatography, Karatepe, 2004) [10]. Fluorimetric determinations of VC have been also developed based on the condensation reactions of VC with o-phenylenediamine, (Wu *et al.*, 2003) [20] and on the oxidation with mercury (II) of VC to form quinoxaline derivate, Perez-Ruiz *et al.*, 2004) [17]. The present study was undertaken to evaluate the comparative efficiencies of some VC quantification processes and also to determine the quantum of VC present in different botanical materials.

Materials and Methods

a) Collection of botanicals

16 botanicals viz., *Emblca officinalis* (amla) fruit and leaves, *Moringa oleifera* (drumstick) leaves, *Carica papaya* (papaya) fruit and leaves, *Citrus lemon* (lemon) peel and leaves, *Psidium guajava* (guava) fruit and leaves, *Citrus sinensis* (orange) fruit, peel and leaves, *Citrus limetta* (sweet lime) fruit, peel and leaves, *Oxalis corniculata* (creeping wood sorrel) plant, *Hibiscus sabdariffa* (rosella) leaves, *Vitis amurensis* (grapes) fruit and leaves, *Brassica oleracea* (cabbage) head, *Solanum melongena* (brinjal) leaves, *Cymbopogon citrates* (lemon grass) leaves, *Ziziphus jujuba* (jujube) fruit, *Solanum lycopersicum* (tomato) fruit and leaves, *Ananas comosus* (Pineapple) fruit were identified and collected.

b) Extraction of botanicals

Fruit pulps, peels and leaves of the collected botanicals were dried in the hot air oven (below 40 °C) and ground to fine powder and used for the extraction. Moisture % of all the plant samples was calculated by the following formula.

$$\text{Moisture \%} = \frac{\text{Fresh weight-dry weight}}{\text{Fresh weight}} \times 100$$

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The powdered plant material was at first thoroughly mixed with chloroform (1:3 w/v) and centrifuged at 8000rpm. The chloroform was made to evaporate from the pallet, to which ether was added (1:3 w/v), mixed thoroughly and again centrifuged. The ether was evaporated from the pallet and the de-etherized pallet was used as sample for extraction of VC. Extraction of the sample was carried out by following m-Phosphoric acid (MPA): acetic acid (AA) extraction method of AOAC (1995) [2].

c) Quantification of botanicals

All the botanical extracts were filtered through Whatman No. 41 filter paper to obtain particle free extract and quantified for VC by the following processes:

Visual Titration

a. Iodine titration

• Titration of standard VC solution

20 ml of VC standard solution and 1 ml of starch indicator solution were poured into a 50 ml Erlenmeyer flask. The iodine titration solution was set up into 50 ml burette on the ring stand. The initial volume of the iodine titration solution in the burette was noted. The Erlenmeyer flask (containing the VC and starch solutions) was placed under the burette. The spring clamp of the burette was carefully released to add iodine solution drop by drop. The flask was swirled to mix the solution after each addition. The titration was considered complete when the iodine created a blue-black color in the solution that lasted for longer than 20 seconds. The final volume of the iodine solution in the burette was recorded. The difference between the initial volume and the final volume was calculated which was the amount of iodine titration solution needed to oxidize the VC. The procedure was repeated three times. The amount of VC needed to reduce 1ml of the iodine solution was calculated and used as constant to calculate the amount of the vitamin in unknown samples.

Amount of VC needed to reduce 1ml of dye was calculated by dividing the amount of VC present in the standard solution by the number of ml of dye titrated.

• Titration of sample solution

Titration of botanical samples were carried out in a quite similar as that of the VC standard titration. 20 ml aliquot of the sample solution was pipetted into a 50 ml conical flask and 1 ml of starch indicator solution was added. The sample was titrated with 0.005 mol/L iodine solution. The endpoint of the titration was identified as the first permanent trace of a dark blue-black colour due to the starch-iodine complex. The titration was repeated with further aliquots of sample solution until concordant results were obtained. The end point of titration in case of plant sample varies according to the colour of the extract used.

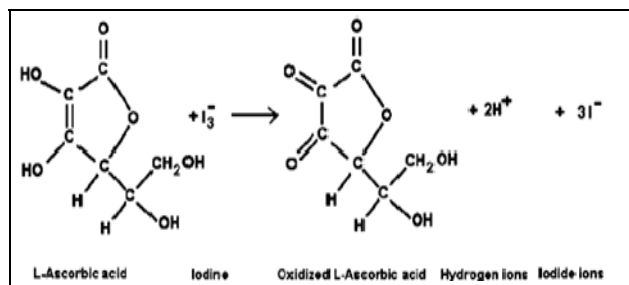


Fig 1: Redox titration of ascorbic acid with Iodine

Calculations

Amount of VC in an unknown plant sample was calculated by the formula

Amount of VC in an aliquot =

Amount of iodine titrated (ml) in aliquot x (mg of VC/ ml of iodine solution).

b. Dichlorophenol-indophenol (DCIP) titration

• Titration of standard VC solution:

The DCIP solution was first standardized against a known amount of VC. This was accomplished by titrating the dye into a solution containing 1.0 ml of VC solution (4.0 mg/ml) and 9 ml of 5% metaphosphoric acid. The end point of the titration was defined as a pink color that persists through at least 15 seconds of swirling. The amount of VC equivalent to 1.0 ml of dye was then calculated.

• Titration of sample solution

Titration of botanical samples were carried out in quite similar as that of the titrating the VC standard. 20 ml aliquot of the sample solution was pipetted into a 100 ml volumetric flask and brought to the final 100 ml volume with the 5% metaphosphoric acid solution. The sample was titrated with 0.08% DCIP solution. The end point of the titration was defined as a pink colour that persisted through at least 15 seconds of swirling. The titration was repeated with further aliquots of sample solution until concordant results were obtained.

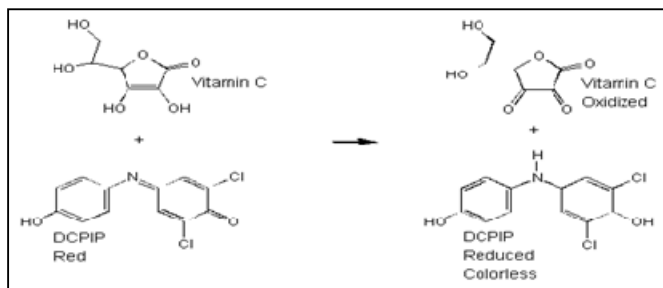


Fig 2: Reduction of DCIP with ascorbic acid

Calculations

Amount of VC in an unknown plant sample was calculated by the same formula used for iodine titration.

Amount of VC in an aliquot =

Amount of DCIP dye titrated (ml) in aliquot x (mg of VC/ml of DCIP dye).

Spectrophotometric determination

Experimental Procedure

Standard VC solution was prepared by dissolving 40 mg of VC in 250 ml of 5% TCA and designated as stock solution. 10 ml of this solution were pipetted out into 100 ml of standard flask and the volume was made up to the mark by adding 5% TCA solution. This solution was kept as working standard solution. A series of 0-1 ml of working standard solution was pipetted out into 6 clean and dry test tubes. The solution in each test tube was made up to 1 ml 5% TCA. 1 ml of 2, 4-dinitrophenyl hydrazine (DNPH) reagent was added to each test tube and latter were boiled for 8-10 minutes in water bath. The test tubes were cooled and 8 ml of 65% H₂SO₄ were added. The solution in each of the test tubes was mixed well and optical density (O.D) was taken at 540nm using Digital Spectrophotometer Model No. 301, Growel Instruments,

Bangalore, India. A known quantity from each of the botanical extracts was taken into clean and dry test tubes in three replications in a series of dilutions. The solution in each test tube was made up to 1 ml with 5% TCA. 1 ml of DNPH reagent was added to each test tube and latter were boiled for 8-10 minutes in water bath. The test tubes were cooled and 8 ml of 65% H₂SO₄ were added. The solution in each of the test tubes was mixed well and optical density (O.D) was taken at 540nm.

Calculations

Amount of VC needed to be read by a unit optical density (OD) was calculated and kept as standard (constant) to measure the amount of VC in an unknown sample. The calculation was directly made using the values of standard VC constant.

Amount of VC in an unknown was calculated as follows.

Amount of VC in an unknown = O.D of sample X (amount of standard VC / O.D)

Table 1: Determination of vitamin C (mg/g dry wt.) in fruit pulps by different quantification methods

Botanical	Quantification method		
	DNPH	DCIP	IODINE
B1	28.340**	19.129**	18.987**
B2	9.643*	6.165*	5.976*
B3	9.567*	6.987*	6.823*
B4	8.695*	4.812*	4.356
B5	8.768*	5.321*	5.654*
B6	7.567*	3.456	4.001
B7	6.455*	2.957	3.689
B8	4.345	2.563	2.987
B9	6.465*	3.579	3.251
SE±	0.427	0.388	0.331
CD 1%	1.914	1.739	1.481

B1: Amla, B2: Guava, B3: Jujube, B4: Orange, B5: Musambi, B6: Papaya, B7: Grapes, B8: Tomato, B9: Pine apple.

** : Highly significant at CD 1%, * : Significant at CD 1%.

Table 2: Determination of vitamin C (mg/g dry wt.) in plant leaves by different quantification methods

Botanical	Quantification method		
	DNPH	DCIP	IODINE
B10	11.987**	8.563**	8.876**
B11	10.870**	7.994**	8.099**
B12	20.437**	12.340**	13.123**
B13	6.580*	3.456	3.123
B14	5.460	2.123	2.234
B15	9.340*	7.654*	7.245*
B16	9.560*	7.156*	6.997*
B17	4.234	2.356	2.765
B18	6.784*	4.876	4.345*
B19	6.345*	4.234	3.987*
B20	4.456	2.879	3.876*
B21	4.743	2.997	2.134
B22	7.001*	3.769	4.124*
SE±	0.329	0.234	0.332
CD 1%	1.475	1.047	1.487

B10: Papaya, B11: Lemon, B12: Drumstick, B13: Amla, B14: Guava,

B15: Creeping wood sorrel, B16: Rosella, B17: Tomato,

B18: Sweet lime, B19: Lemon grass, B20: Brinjal, B21: Cabbage,

B22: orange, B23: Grapes.

Table 3: Determination of vitamin C (mg/g dry wt.) in fruit peels by different quantification methods

Botanical	Quantification method		
	DNPH	DCIP	IODINE
B23	11.765**	7.123	6.446
B24	9.987	6.567	5.982
B25	9.564	6.784	5.643
SE±	0.268	0.409	0.276
CD 1%	1.202	1.833	1.235

B24: Lemon, B25: Orange, 26: Sweet lime.

Results and Discussion

In the present study, two protocols *viz.*, spectrophotometric (DNPH) and titrimetric (Iodine and DCIP) were evaluated for their efficiencies of VC quantification using 16 botanicals. Results were validated by performing a comparative VC recovery study. The data on the quantum of VC/g dry of 26 parts of 16 botanicals are presented in Tables, 1-3. Spectrophotometric quantification method (DNPH) revealed maximum quantum of VC in all the 16 botanicals as compared to titrimetric (iodine and DCIP) method. Whereas, the quantum of VC measured by DCIP and Iodine method was almost at parity with each other. Among the 9 fruit pulps (B1-B9), the highest value of VC mg /g dry wt. was recorded in case of amla fruit pulp (28.340) followed by guava (9.643), jujube (9.567), orange (8.695), sweet lime (8.768), papaya (7.567), pine apple (6.465), grapes (6.455) and tomato (4.345) (Table 1). Drumstick leaves yielded highest content of VC (20.437 mg/g dry wt.) among the 14 different leaves used (B10-B23). Papaya leaves recorded 11.987 mg/g dry wt. followed by lemon (10.870). Other leaves *viz.*, rosella, creeping wood sorrel, grape, orange, sweet lime, amla, lemon grass, guava, cabbage, brinjal and tomato showed comparatively lesser content of VC as 9.560, 9.340, 9.234, 7.001, 6.784, 6.580, 6.345, 5.460, 4.743, 4.456 and 4.234 mg/dry wt. (Table 2) respectively. Among the three fruit peels used, lemon yielded the maximum VC content (11.765 mg/g dry wt.) followed by orange (9.987 mg/g dry wt.) and sweet lime (9.564 mg/g dry wt.) (Table 3).

In brief, the highest value of VC mg /g dry weight (28.340) was recorded in amla fruit pulp. Drumstick leaves stood second in line after amla, recording 20.437 VC mg /g dry weight. Papaya leaves was the third highest VC rich (11.987) botanical followed by lemon peel (11.765) and lemon leaves (10.870) among the all botanicals screened.

A wide range of methods of VC determination can be found in literature on the subject and a considerable part of them is based on its reduction properties. Among the titrimetric methods iodine and DCIP are applied commonly. However, these methods do not allow determining the dehydroascorbic acid (DHA), which is characterised by the same vitamin activity as the ascorbic acid (Moszczynski & Pyc, 1999) [14]. In addition, titration methods are not sufficiently selective as reduction substances in food products interfere with the process of VC determination and distort the obtained results (Arya *et al.*, 2002) [3]. On the other hand, spectrophotometric method (DNPH), the one used in present study, provides more selective possibilities of vitamin C determination of the sum of ascorbic acid and dehydroascorbic acid. This method allows a simultaneous determination of both ascorbic acid and dehydroascorbic acid and is therefore more reliable compared to DCIP and iodine methods. Perusal of the tables 1-3 reveals more VC measurements in all the botanicals by DNPH method, witnessing the determination of total vitamin C

compared to the titrimetric methods which measure only one part of it (ascorbic acid). These results are in agreement with those obtained by Klenner (1974) [12] who put forth that blood and urine samples analyzed with DCIP will give values roughly 7% less than testing with DNPH. Esteban and Ho (1997) [6] determined VC by spectrophotometric method in a variety of samples. VC content as measured by iodine and DCIP methods in lemon, grape and cabbage did not differ significantly from each other in the present study. These observations are in the line of observations made by Bessey and King (1933) [4]. They quantitated the lemon, grape fruit, pepper (ripe and green), cabbage, rhubarb and green beans (fresh and canned) by iodine and DCIP and reported that the VC measurements in case of iodine did not differ significantly from those obtained for DCIP. Quantum of VC in any botanical varies according to the botanical type, temperature, season and maturity stage. The content of this vitamin in fruits and vegetables varies between cultivars and tissues (Lee and Kader, 2000) [13]. In the present study, VC content of 4.356, 6.446 and 3.689 mg /g dry weight was recorded in orange, lemon and grape fruits respectively using iodine method (Table 1). Izuagie and Izuagie (2007) [9] determined iodimetrically (iodine method) the VC content of the juices of four different citrus fruits - orange, tangerine, grape fruit and lime. Results showed that orange had the highest value of VC, 600 µg /ml followed by grape, 446 µg /ml and then tangerine, 415 µg /ml. Lime had the least value, 306 µg /ml. The amount of VC as estimated by iodine method in present study with regard to guava fruit and amla fruit pulp are 5.976 and 18.987 mg/g dry weight. Suntornsuk (2002) [19] measured the vitamin C content in fresh and freeze-dried herbal juice, such as guava (*Psidium guajava* Linn.) amla (*Emblica officinalis*), lemon (*Citrus lemon*), sweet pepper (*Capsicum annum*) and passion fruit (*Passiflora laurifolia*) by direct titration with iodine. The method showed excellent linearity ($r^2 > 0.99$) over the concentration ranges tested (100–500% of the amount found in the juice samples), good precision and recovery. The limits of detection and quantitation were 2.2 and 7.3 mg respectively. The amount of VC found were 80.1 mg/100 g for guava, 226.0 mg/100 g for amla, 52.8 mg/100 g for sweet pepper, 39.1 mg/100 g for passion fruit, 10.5 mg/100 g for lemon and 4.6 mg/100 g for *G. schomburgkiana*.

Many dyes such as 2,6-dichlorophenolindophenol(DCIP), dimethoxydiquinone (DMDQ), ninhydrin, fast red AL salt and 2, 7-dichloroflourescein *etc.* have been used for the estimation of VC. Among these dyes DCIP has been most extensively studied. It is included in the official titrimetric methods as reported in different pharmacopoeias (U.S. Pharmacopoeia, 1970, Indian Pharmacopoeia, 1985 and British Pharmacopoeia, 1988) [22, 10 and 5] and also forms the basis for many colorimetric methods. In the present study, amount of VC as determined by iodine and DCIP method was 18.987 and 19.129 mg/g dry weight in case of amla, whereas, Khopde *et al.*, 2001 estimated the VC content in the amla extract by following DCIP method to the extent of 4.465% or 44.65 mg/g of amla. The DCIP method for the determination of vitamin C has been extensively used in various laboratories. Agbo (2005) [1] measured the VC in potato by DCIP and revealed that the potato chips contained higher mean VC (10.9mg VC/100g) than the boiled pieces (6.6mg VC/100g).

It has been observed after reviewing the previous findings of different authors with regard to the quantification of VC that, it varied according to the type of plant, maturity *etc.*, and even

totally contradictory reports also have been published. The differences in VC content as reported by many workers could be attributed to different factors such as variety, temperature, pre-conditioning, handling, storage temperature and storage duration (Lee & Kader, 2000) [13]. Further, DHA does not account for more than 10% of total vitamin C in any of the analyzed fruits as has been described by Lee & Kader (2000) [13]. It has been noted that when reporting vitamin C levels, many researchers have not taken into account DHA. More VC content in lemon leaf (20.437mg/g dry wt) than in its fruit peel (11.765mg/g dry weight) observed in the present work is in accordance with the finding of Smirnoff *et al.*, (2001) [18]. According to them different plant species and tissues have characteristically different VC concentrations and in leaves the VC pool size is light dependent. Leaf tissue contains more VC as compared to the fruit peel tissues because the leaf tissue is more exposed to oxidative stresses. VC is very important in photoprotection and in the regulation of photosynthesis (Noctor and Foyer 1998) [16]. Higher photosynthesis is found in leaves and hence it is likely that leaves do have more ascorbate than fruit peel tissue. The photosynthetic role in fruit peel tissues are extremely low or non existent.

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