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Ascorbic acid quantification in *Benincasa hispida* fruit extracted using different solvents

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Abstract

Ascorbic acid or vitamin C is mostly found in natural products such as fruits and vegetables. High performance liquid chromatography (HPLC) method has been developed and validated to compare the ascorbic acid content in *Benincasa hispida* (Bh) fruit extract with three different extraction solvents; i) 3% metaphosphoric acid, ii) 3% citric acid and iii) distilled water. The compound has been detected and quantified by the use of HPLC coupled with UV-Vis detector. The amount of ascorbic acid detected in Bh fruit extract prepared with different extraction solvents; 3% metaphosphoric acid, 3% citric acid and distilled water were 13.18, 7.91 and 9.42 mg/100g respectively. Total run time was 6 min and the retention time was 2.60 min. Calibration curve was linear with the concentration range 1.00 – 16.00 µg/ml. Limits of detection was 0.24 µg/ml, limit of quantification was 0.81 µg/ml and recovery was 93.52%. The result showed ascorbic acid content is higher in Bh fruit extract with 3% metaphosphoric acid, followed by extract with distilled water and 3% citric acid. Thus, Bh is another novel fruit/vegetable potentially used as food ingredient as it contains a good source of ascorbic acid that can be good for one's health.

Introduction

Benincasa hispida (Bh) commonly known as ash gourd or winter melon is from the Cucurbitaceae family. It has been valued as a healthful vegetable as it is good source of natural sugars, amino acids, organic acids, mineral elements and vitamins (Zaini *et al*., 2010). Nutritionally, Bh fruit contains major contents of water with minor amounts of protein, fat and ash. Edible portion of Bh flesh recorded the highest vitamin C and riboflavin content. Previous study showed the presence of triterpenes, catechins, carotenes, tannins, uronic acids and polyphenols in Bh extracts (Gill *et al.*, 2010)

Fruits have large amounts of potentially interfering compounds. Analysis of ascorbic acid (Figure 1) from specific plant tissue types need great caution in the use of methods that have been developed (Davey *et al*., 2000). Ascorbic acid will be oxidized under alkaline conditions. The use of a high ionic strength, acidic extraction solvent is required to block metabolic activity upon disruption of the cell and to precipitate proteins.

L-Ascorbic acid, also known as vitamin C (C_6H_8O) is widely distributed in nature, typically rich in fresh fruits and leafy vegetables such as guava, mango, papaya, cabbage, mustard leaves and spinach (Tee *et al.*, 1997). Ascorbic acid is an antioxidant but it is the least stable of all vitamins and is easily oxidized during processing and storage. Juices are good foods to be fortified with ascorbic acid because their acidity reduces ascorbic acid destruction. Exposure to oxygen, prolonged heating in the presence of oxygen, and the presence of oxidizing enzyme, contact with minerals (iron and copper) and exposure to light are destructive to the ascorbic acid content of foods. Dehydroascorbic acid is an oxidized form of ascorbic acid. In this study, only ascorbic acid was measured since at harvest, dehydroascorbic acid represents less than 2% of total vitamin C (Wills *et al*., 1984).

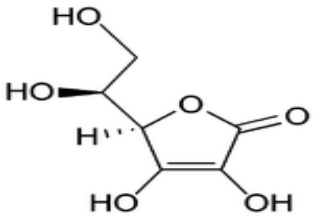

Figure 1. Chemical structure of ascorbic acid

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Normally, the method used to determine ascorbic acid is based on the reduction of the blue dye 2,6 dichlorophenolindophenol by ascorbic acid (AOAC, 1999). The endpoint of the titration is indicated by the appearance of the pink acid form of the dye. This is a simple and fast method (De Assis *et al*., 2001). Alternatively, analysis of ascorbic acid by HPLC allows the determination of ascorbic acid in an easy, fast, and precise method. HPLC is considered a sensitive and selective method and therefore suitable for active substance determination; it is also suitable for the evaluation of stability in formulations in the pharmaceutical and cosmetic industries (Marshall *et al*., 1995). In addition, some studies reported liquid chromatography can avoid the problem of non-specific interference and ion-pair (Ke *et al.*, 1994) NH2 bonded-phase (Silva, 2005) and reverse phase (Franke *et al.*, 2004) techniques. The purpose of this study was to determine, develop and validate a HPLC method for the quantitation of ascorbic acid in Bh extract in different extraction solvents.

Materials and Methods

Chemicals

L-ascorbic acid, metaphosphoric acid and citric acid were purchased from Sigma, USA. Potassium dihydrogen phosphate (KH2PO4) was purchased from Merck, Germany.

Standard preparation

Ascorbic acid (10 mg) was weighed accurately and transferred to 100 ml volumetric flask. The standard was dissolved in 100 ml HPLC grade water to prepare standard stock solution of 100 µg/ml.

Sample preparation

The technique of extraction was a simulation of the method performed by Albuquerque *et al.* (2005). The fresh fruit of Benincasa hispida was cut into small pieces after removing seeds and peels. 20 g of the fruit was homogenized with 30 ml extraction solvent and centrifuged at 15000 g for 25 min at 4oC. Then, the slurry was filtered through Whatman No. 4 followed by membrane Miliphore (0.45µm) prior to injection into HPLC system. There were three extraction solvents involved in this investigation; i) 3% metaphosporic acid, ii) 3% citric acid, iii) distilled water.

HPLC-UV method

Separation of ascorbic acid was performed by HPLC with a Gilson UV/Vis 151 Series HPLC system (operated at 230 nm) and injection valve with 20 µl sample loop. The compound was separated on 125 mm × 4 mm, i.d., 5-µm pore size LiChrospher 100 RP-18 column (Merck, Germany). The mobile phase, prepared using 0.02M potassium dihydrogen phosphate, was filtered through a 0.45 µm. The flow rate was 0.5 ml/min. Data was integrated by Trilution LC software and results were obtained by comparison with standards.

Calibration plots

Solutions of ascorbic acid standard with different concentrations were prepared by dilution of the standard solution. The standard response curve for ascorbic acid was a linear regression fitted to triplicate values obtained at each of five concentrations (1.00 to 16.00 µg/ml). Precisely 20 µl of each solution was chromatographed and the peak areas were measured. Peak areas against the respective concentration for ascorbic acid were then plotted to find the linear range of ascorbic acid.

Method validation

The method was validated for repeatability, linearity, accuracy, precision, selectivity and specificity. Accuracy was assessed by spiking standard of ascorbic acid in the three different samples. Measurements of recoveries were also done. The precision was measured by intra- and inter-day relative standard deviation (R.S.D) of peak area and retention time. All validation studies were performed by replicates injection of standard and sample solutions. For intra-days test, the concentrations of the compound were calculated three times on the same day at intervals of 1 hour whereas for inter-day study, the concentrations of the compound were calculated on three different days. Selectivity and specificity of the method were assessed by injecting solutions containing the standard. LOD and LOQ were measured to estimate the detection and quantitation limits of the method. It was calculated using the equation LOD = 3 σ/S; LOQ = 10 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve.

Results and Discussion

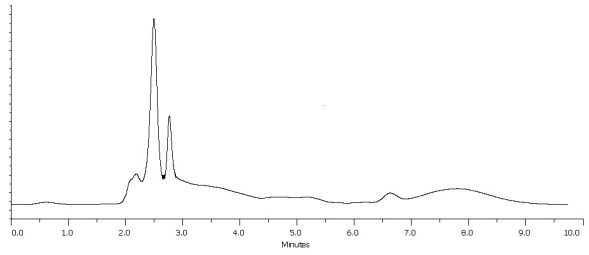
Concentration of ascorbic acid in Bh extracted with different solvents

The present result showed Bh extracted with different solvents recorded different concentration after quantification was accomplished using HPLC (Table 1). *Benincasa hispida* extracted using 3% metaphosphoric acid recorded the highest concentration of ascorbic acid (13.18 mg/100 g) significantly higher than distilled water (9.42 mg/100 g) and 3% citric (7.91 mg/100g). This indicated that

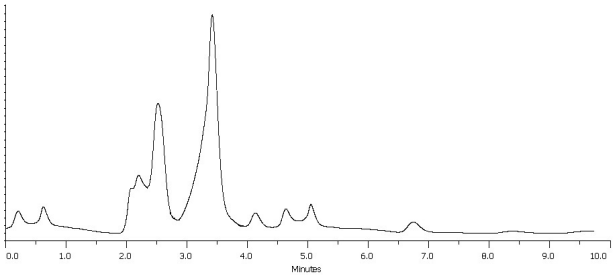
Table 1. Concentration of ascorbic acid from *Benincasa hispida* (Bh) prepared with three different solvents (n=3)

Sample	Ascorbic acid (mg/100g)
3% metaphosphoric acid	13.18[a]
3% citric acid	7.91[c]
distilled water	9.42[b]

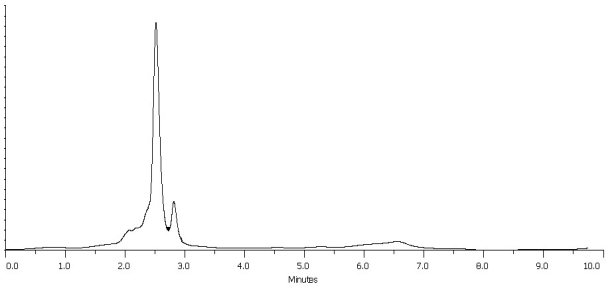
[a-c] Mean values in the same column with different letters are statistically different (p<0.05)



a)Chromatogram of Bh fruit extracts with 3% metaphosphoric acid



b) Chromatogram of Bh fruit extracts with 3% citric acid



c) Chromatogram of Bh fruit extracts with distilled water

Figure 2. Chromatogram of ascorbic acid in Bh fruit extracted with different extraction solvents

metaphosporic acid is an efficient solvent in extracting ascorbic acid from Bh. This situation confers the fact that metaphosphoric acid may provide proficient ascorbic acid extraction by preventing oxidation (Franke *et al*., 2004) compared to other acidic solvents.

In addition, the result indicates that the efficiency of extraction was higher in 3% metaphosphoric acid, followed by distilled water and 3% acetic acid. However, the concentration of ascorbic acid was found to be not significant in banana, papaya, and mango when the extraction was done using some selected extractants namely metaphosphoric acid, acetic acid and oxalic acid (Hernández *et al*., 2006). The difference may be due to the fact that the previous study (Hernández *et al*., 2006) emphasized on climacteric fruits while the present work focused on the non-climateric fruit. Adding metaphosphoric acid to the extracting solution contributed to ascorbic acid protection during the extraction process because ascorbic acid is easily oxidized under alkaline conditions. In addition, it was an indispensable measure in the case of vegetable analysis (Campos *et al*., 2009) because the vitamin is easily oxidized during extraction process especially in natural products. Besides, approximately all tissues containing vitamin C also contain one or more enzymes which catalyze its aerobic oxidation to a marked degree as soon as the cellular tissue is crushed or severely damaged (Szent-Gyorgyi, 1928).

Meanwhile, ascorbic acid content of Bh extracted with 3% citric acid in the present study was lower and contradicts with the result reported by Albuquerque *et al*. (2005). This group suggests the use of 3% citric acid as an extraction solvent to quantify ascorbic acid for melon samples in HPLC analysis. This difference may be due to incompatibility of extraction mediums, different types of samples and HPLC parameters used during analysis. Variability in phytonutrients occurred in both Benincasa hispida and melons can possibly be an additional factor that purported to cause this difference.

From the present study, the mobile phase conditions were optimized thus there was no interference from solvent and other compounds. It consisted of isocratic elution of 0.02 M potassium dehydrogen phosphate (KH_2PO_4) in HPLC-grade water which was found to be a suitable mobile phase allowing good separation of ascorbic acid at flow rate 0.5ml/min using LiChrospher 100 RP-18 column. Under this system, the ascorbic acid in both Bh fruit extracts and standard was able to be detected. The chromatogram of both standard and sample are shown in Figure 2. Using this system, the ascorbic acid was clearly eluted with retention time of 2.60

Table 2. Validation data from calibration curves of ascorbic acid in Bh fruit extracts

Retention time	Regression equation	Correlation coefficient (R)	Linear range (µg/ml)	Detection limit (µg/ml)	Quantification limit (µg/ml)
2.60	y = 112358x - 11367	0.9999	1.00 – 16.00	0.24	0.81

Table 3. Concentration of ascorbic acid recover from Bh fruit extracts in different extraction solvent (n=3)

Bh extract in different extraction solvent	Amount added (µg/ml)	Amount found (µg/ml)	Recovery (%)	Average Recovery (%)
3% metaphosphoric acid	8	7.05	88.18	
3% citric acid	8	6.40	80.03	93.52
distilled water	8	8.99	112.38	

Table 4. Concentration of ascorbic acid recover from Bh fruit extracts in different extraction solvent (n=3)

Intra-day R.S.D. for Retention time (%)	Intra-day R.S.D for Peak area (%)	Inter-day R.S.D for Retention time (%)	Inter-day R.S.D for Peak area (%)
0.25	2.33	0.21	3.81

min.

Ascorbic acid is an essential water-soluble vitamin that is necessary for normal growth and development but it is easily destroyed and least stable. Due to its sensitivity, it is hard to quantify ascorbic acid amount in natural samples so extraction solvent may contribute an important role in determining ascorbic acid. Different extraction solvents of Bh fruits extract can distinguish the amount of ascorbic acid found in the extract. It can be analyzed from HPLC analysis by superimposing the peak of ascorbic acid with peaks of samples with absorption at 230nm.

Albuquerque *et al.* (2005) who quantified ascorbic acid in melon samples revealed the importance of extraction medium for HPLC analysis. Furthermore, auto-oxidation of ascorbic acid by oxygen is greatly decreased by an acidic medium which is necessary to stabilize ascorbic acid (Wimalasiri and Wills, 1983).

Validation of the method

The method was validated for linearity, accuracy, precision, selectivity and specificity. All validation studies were performed by replicate injection of standard and sample solutions. Standard solutions at five different concentrations were analyzed and calibration plots were constructed by plotting mean areas against respective concentrations. The method was assessed by determination of the correlation coefficient and intercept values as shown in Table 2. From the calibration plot, the linear range for ascorbic acid can be observed clearly ranging from 1.00-16.00 µg/ml with the correlation coefficient of 0.999. In addition, both detection limit and quantification limit were at 0.24 µg/ml and 0.81 µg/ml, respectively.

Recovery study has been conducted to confirm the accuracy of the method developed. Standard solution (10 µg/ml) was added into a three different pre-analyzed sample solutions and the recovery of the compound was calculated. The results of accuracy test were shown in Table 3. The present accuracy test shows that ascorbic acid of Bh extracted with 3% metaphosphoric acid, 3% gallic acid and distilled water recorded recovery level at 88.18, 80.03 and 112.38%, respectively. It was also shown that the present method enables accurate quantitative analysis of ascorbic acid in three different Bh extracts. In addition, the average of recovery levels for all spiked ascorbic acid standard in all samples was successfully recovered at 93.52%.

The precision aspects were assessed by measuring the intra and inter-day of retention time and peak area for ascorbic acid standard by replicates analysis. It was expressed as the relative standard deviation (R.S.D) (Table 4). The present results show that intra and inter-day R.S.D for retention time and peak area were both low and the precision was acceptable. The intra-day R.S.D for retention time was 0.25% while the intra-day R.S.D for peak area was 2.33%.

Meanwhile, the inter-day R.S.D for retention time was recorded at 0.21% with the inter-day R.S.D for peak area was 3.81%.

The selectivity of the method for the standards was established by study of the resolution between the standards peak. Under the chromatographic conditions, the peak of ascorbic acid was completely separate and no interference. This indicates that the method was quite selective. The specificity was evaluated by comparing the chromatograms obtained from extracts and from the standard as shown in Figure 2. Because of the retention times of standard solution and Bh fruit extracts were identical and no co-eluting peaks from the diluents were observed, the method was specific for quantitative estimation of ascorbic acid in this plant extract.

Conclusion

Determination of ascorbic acid in Benincasa hispida extracted with different mediums by HPLC-UV represented an excellent technique with high sensitivity, precision and reproducibility. The method gives a good resolution for ascorbic acid with an isocratic elution. The method able to detect the occurrence of ascorbic acid in Bh extracted with different solvents. Metaphosphoric acid was recommended as a suitable extraction solvent for ascorbic acid in Bh fruit compared to acetic acid and distilled water. Thus, Bh is another novel fruit/vegetable potentially used as food ingredient as it contains high amounts of ascorbic acid.

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