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HPLC Determination of Ascorbic Acid and Dehydroascorbic Acid in Chinese Jujube

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A high-performance liquid chromatographic method for determination of ascorbic acid and dehydroascorbic acid in Chinese jujube was developed. The dehydroascorbic acid content in sample can be calculated by the difference between the total ascorbic acid (after dehydroascorbic acid reduction) and the ascorbic acid concentrations (before reduction). The method resulted linear up to 42 mg/L with precision of 1.09 % and mean recovery of 103.4 %. The least limit of detection and quantitation were 0.02 and 0.11 mg/L, respectively. The 0.1 % H_3PO_4 solution was opted as extracting solvent after analyzing the effect of pH on the efficiency of ascorbic acid extraction. Using of HPLC method, the ascorbic acid and dehydroascorbic acid contents in fruits of six cultivars of Chinese jujube were analyzed. The contents of ascorbic acid and dehydroascorbic acid varied from 225.10 to 424.74 mg/100g and 27.03 to 103.62 mg/100g, respectively, which revealed that fruits of Chinese jujube were good sources of both ascorbic acid and dehydroascorbic acid.

Key Words: Chinese jujube, Ascorbic acid, Dehydroascorbic acid, High-performance liquid chromatography.

INTRODUCTION

Vitamin C is defined as the generic term for all compounds exhibiting the biological activity of ascorbic acid[1]. Generally, the total vitamin C in fruits is assumed to be the sum of both ascorbic acid and its first oxidized form dehydroascorbic acid (DHAA)[2]. They play a vital role in human health. The ratio of ascorbic acid and dehydroascorbic acid can be an indicator of the redox state of an organism[3,4]. Ascorbic acid is essential for the synthesis of collagen and intercellular material, the enhancement of the immune system, absorption of iron and prevention of cardiovascular disease through its free radical-scavenging activities[5-9]. Dehydroascorbic acid is also an important and interesting but somewhat enigmatic compound in biological systems[10,11]. It can be easily converted into ascorbic acid in the human body, and exhibits biological activity[12-14]. On the other hand, dehydroascorbic acid reacts with homocysteine thiolactone converting it into the toxic compound, 3-mercaptopropionaldehyde which kills the cancer cells, while ascorbic acid has no effect[15].

Numerous analytical methods to quantify the amount of vitamin C in biological samples have been reported in literature. These include titrimetric method[16], spectrophotometry[17-19], calorimetry[20], chemiluminescence[21], voltammetry[22,23], enzymatic assays[24,25], amperometric method[26,27] and high-performance liquid chromatography (HPLC)[2,28]. Most of these methods, other than HPLC, are time-consuming and may give overestimates due to the presence of oxidizable components other than ascorbic acid and/or not to measure dehydroascorbic acid[29,30]. Dehydroascorbic acid is difficult to be detected because of its low UV absorbivity even using HPLC method[30]. In order to determine its content, it is important to reduce dehydroascorbic acid to ascorbic acid in samples[3]. Usually, the dehydroascorbic acid content in samples can be calculated by the difference between the total ascorbic acid (after dehydroascorbic acid reduction) and the ascorbic acid concentrations (before reduction)[31,32]. Various reducing agents, such as homocysteine[31], dithiothreitol (DTT)[29,30] and cysteine[1] have been studied.

Chinese jujube (*Ziziphus jujuba* Mill.) is a native fruit tree of China and has been introduced into more than 40 countries throughout the world. It is commercially cultivated mainly in China and South Korea with a total growing area of over 1,600,000 hectares. Its fruit is not only a good-tasting food but also an important traditional Chinese medicine. It is famous for high vitamin C content (200-800 mg/100 FW) which is even higher than in kiwi fruit. On the average, a 20 g portion of Chinese jujube fruit studied would provide about all of an adult's daily need of vitamin C[16]. In general, the vitamin C content of Chinese jujube is measured by the visual titrimetric method of reduction of 2,6-dichloro indophenol (DCIP). Strictly, what is determined by this method is ascorbic acid content instead of the total vitamin C. That is not sufficient

when reporting total vitamin C levels. While, there is a clear lack of knowledge of dehydroascorbic acid in Chinese jujube. In this paper, a simple and rapid HPLC method for the determination of ascorbic and dehydroascorbic acid in Chinese jujube was firstly developed, in which the dehydroascorbic acid was converted into ascorbic acid using dithiothreitol as reducing agent. The reliability of the method was evaluated in terms of linearity, limit of detection (LOD) and limit of quantification (LOQ), precision and accuracy. Using this method the contents of ascorbic acid and dehydroascorbic acid in the fruits of six Chinese jujube cultivars were determined. The HPLC method for ascorbic acid determination was then compared with the conventional visual titrimetric method of reduction of 2,6-dichloro indophenol. In addition, the effect of pH on the efficiency of ascorbic acid extraction and the stability of ascorbic acid before and after the incubation with dithiothreitol were studied.

EXPERIMENTAL

Six important cultivars of Chinese jujube including Jinsixiaozao, Zanhuangdazao, Huizao, Junzao, Pozao and Maoboyan were employed for analyzing ascorbic acid and dehydroascorbic acid contents in this paper. The first five cultivars were collected from National Jujube Repository located in Taigu, Shanxi, China, and the last one was from Jujube Repository of Agricultural University of Hebei located in Xianxian, Hebei, China. The sample fruits were harvested at physiological maturity stage and stored at -70 ºC.

The analyses of ascorbic acid were performed by an Agilent 1200 model HPLC system (Waldbronn, Germany) consisting of a quaternary pump with vacuum degasser, a temperature controlled column oven set at 25 ºC, a UV-visible detector (UV) set at 260 nm. The chromatographic separations were performed on a Venusil MP-C18 column (Agela, 250 mm × 4.6 mm i.d., 5 μm). A 0.02 M phosphate buffer (pH5.8) was used as the mobile phase with a flow-rate of 1 mL/min. The standard solutions and sample extracts were filtered through a 0.45 μm Millipore disposable filter before injection in the chromatography. Ascorbic acid peak was identified by comparing retention time with a commercial standard of ascorbic acid.

Analysis of vitamin C: Chinese jujube fruit samples were cutted into small pieces. A portion of 5 g of fruit was added to 10 mL of 0.1 % H_3PO_4 solution. The mixture was homogenized by grinding, transferred to a 100 mL volumetric flask and diluted to volume. After centrifuging at 10,000 g (refrigerated at 4 ºC) for 10 min, a 10 mL aliquot of supernatant was transferred to a 100 mL volumetric flask and diluted to volume. The sample was used to estimate ascorbic acid and dehydroascorbic acid separately.

Ascorbic acid assay: A 20 mL sample was diluted with equivalent ultrapure water, and then was analyzed by HPLC system and the visual titrimetric method of reduction of 2,6-dichloroindopheno, separately.

Dehydroascorbic acid assay: Another 1 mL sample was diluted with equivalent 0.1 g/mL aqueous solution of dithiothreitol and then kept in the dark at 25 ºC for 0.5 h to convert any dehydroascorbic acid into ascorbic acid. After conversing dehydroascorbic acid into ascorbic acid completely, the sample was analyzed for its total ascorbic acid (ascorbic acid + dehydroascorbic acid) by HPLC. The concentration of dehydroascorbic acid is calculated by subtraction.

Analyses of all samples were run in triplicate. To prevent the loss of ascorbic acid, it was necessary to protect the standard solutions and the samples from light by using amber flasks.

Statistical analysis: Data analysis was carried out with Systat statistical program version 16 (SPSS Inc, USA). The results are presented as means ± SD (standard deviation) (n = 3). Data were analyzed by the one-way ANOVA. Analysis of variance was used to evaluate the different cultivars of Chinese jujube. Fisher's Least-Significant-Difference test (LSD) was applied to experimental results to assess intra-pair significant differences ($p < 0.05$).

RESULTS AND DISCUSSION

Chromatography optimization: Chemical and physical variables were optimized to reduce the analysis time while keeping a good resolution between the peaks of ascorbic acid and other co-extracted compounds in the samples. The best flow rate was 1 mL/min (optimized between 0.7 and 1.5 mL/min). The oven temperature was studied over the range 15-50 ºC and 25 ºC was considered optimum because there were ascorbic acid losses at higher temperatures and there were higher column pressure at lower temperatures. In order to choose the best wavelength for the HPLC analysis, UV scanning was carried out using an ascorbic acid standard. The maximum absorbance was found to be 260 nm (Fig. 1). The concentration of phosphate buffer was studied over the range 0.005-0.05 M. Good results were obtained when using 0.02 M phosphate buffer (pH 5.8) as the mobile phase.

Typical chromatograms (Fig. 1) were shown for the elution of a standard solution of ascorbic acid alone and ascorbic acid in 'Jinsixiaozao' before and after the incubation with dithiothreitol. The retention time for ascorbic acid was 3.027 min. Therefore this HPLC procedure was very rapid and the peak of ascorbic acid had no remarkable change after adding dithiothreitol in sample.

Method validation: The following parameters were determined: linearity, limit of detection and limit of quantification, precision and accuracy. Calibration equation for ascorbic acid was constructed by plotting the UV response against the ascorbic acid concentration at seven concentration levels. UV response (y) of ascorbic acid were significantly linear up to 42 mg/L (y = 46.475x - 20.583) with a regression coefficient (r^2) of 0.9998. The limit of detection and quantitation were calculated as 3 and 10 times the standard deviation of the blank signal, respectively. For ascorbic acid determination, these were 0.02 and 0.11 mg/L, respectively. The precision of the extraction method was determined by repeatability and was expressed as the relative standard deviation (RSD) of replicate measurements. The result was 1.09 % and met the acceptable precision standards. The accuracy was calculated on the recovery of known amounts of analyte, spiking analyte in 'Jinsixiaozao' samples. For estimating the ascorbic acid levels, 'Jinsixiaozao' samples were spiked with ascorbic acid at three fortification levels, 50, 100 and 200 % of the estimated initial

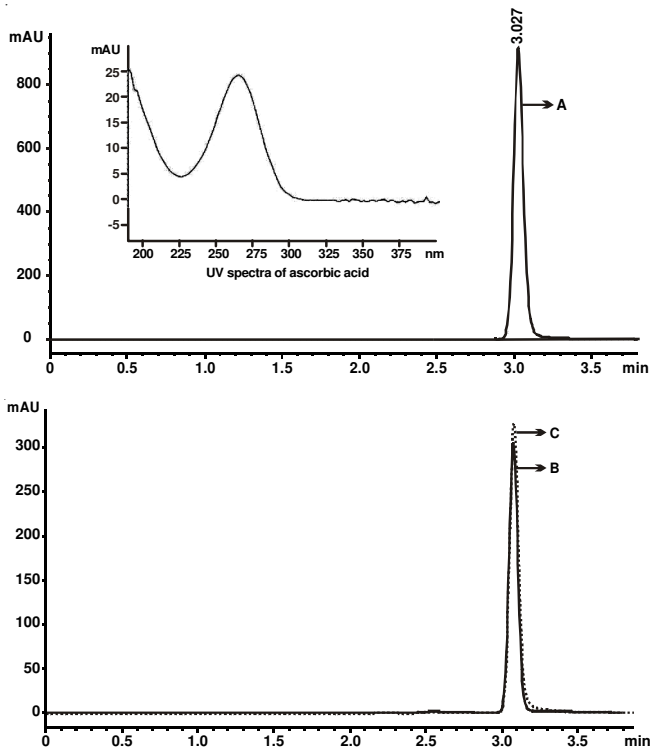

Fig. 1. Chromatograms of standard ascorbic acid (A), ascorbic acid in the extract of 'Jinsixiaozao' before reduction (B) and ascorbic acid in the extract of 'Jinsixiaozao' after reduction of dehydroascorbic acid with dithiothreitol (C). The difference (C minus B) represents the amount of dehydroascorbic acid in 'Jinsixiaozao'

ascorbic acid amount. The recovery percentages in each instance were 103.6 ± 1.17, 103.9 ± 2.45 and 102.7 ± 2.38 %, respectively, and the mean recovery was 103.4 %. The results were satisfactory for ascorbic acid measurement using the HPLC techniques.

Reduction reaction efficiency: As dehydroascorbic acid standard was not commercially available, it was obtained through the oxidation of ascorbic acid stock solution by activated carbon[29,30,33].

The reduction of dehydroascorbic acid to ascorbic acid was catalyzed using dithiothreitol and depended on pH, temperature and reaction time. The reduction followed a zeroth order kinetics pattern[29,30]. The optimal conditions for this reduction were studied by Okamura, who found an optimal pH of 6.5-8.0[34,35]. But, under this condition the stability of ascorbic acid in Chinese jujube was significantly decreased. Thereby this reduction reaction was completed in acid solutions. In addition, the efficiency of the conversion of dehydroascorbic acid into ascorbic acid was studied. The time that complete reduction required depends on how much the amount of dithiothreitol added. When 1 mL 0.1 g/mL dithiothreitol in water solution were added into 1 mL 0.01 mg/mL dehydroascorbic acid in 0.1 % H_3PO_4 solution, the complete conversion could be achieved in 0.5 h (Fig. 2). The reaction was carried out at 25 ºC in a dark place.

Sample preparation efficiency: Most of the methods proposed to use acid solutions as the extracting solvent in order to stabilize ascorbic acid. Acids commonly used were metaphosphoric, citric, perchloric, acetic and orthophosphoric acids. The stability of ascorbic acid is more affected by the

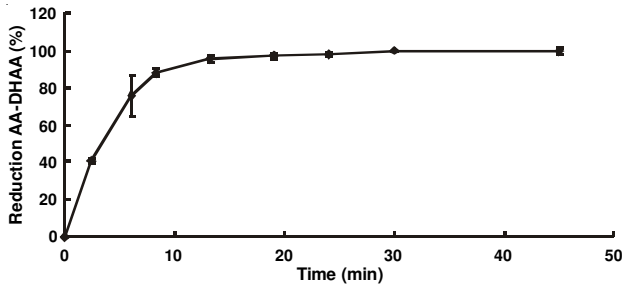

Fig. 2. Conversion (%) of dehydroascorbic acid (DHAA) into ascorbic acid (AA) with 0.1 g/mL dithiothreitol (DTT)

solution acidity than by the nature of the added acid[36,37]. At the same time, Kall *et al.*[36] noted that orthophosphoric acid is suitable to stabilize ascorbic acid and may cause lesser damage to HPLC system. In this study, effect of pH on efficiency of ascorbic acid extraction using Jinsixiaozao samples was studied. The results were shown in Fig. 3. The pH of 2.1-3.0 and 6 were found to be optimal and not significantly different. So, the 0.1 % H_3PO_4 (pH 2.3) was opted as extracting solvent. In addition, the standard preparation and sample solution preparation (Jinsixiaozao) of ascorbic acid without dithiothreitol were found to be stable within 60 and 50 min at 25 ºC, respectively, and both kept stable within no less than 6 h at 4 ºC. ascorbic acid formed by dithiothreitol was stable for at least 1 h in the dark in both solutions at 25 ºC. Since this reaction was completed at 25 ºC, the stability wasn't studied at 4 ºC. It was recommended to measure the total ascorbic acid just after completion of the reaction (0.5 h), because the stability of ascorbic acid might be lower in different cultivars in which oxidation potential is higher.

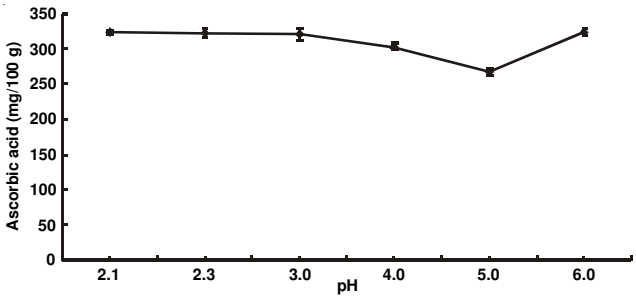

Fig. 3. Effect of the pH on the extraction efficiency of ascorbic acid

Comparison between the HPLC method and titrimetric method for ascorbic acid determination: Ascorbic acid contents of the six cultivars of Chinese jujube were determined by the HPLC method and the visual titrimetric method of reduction of 2,6-dichloroindopheno, respectively. The results were shown in Table-1. The contents determined with titrimetric method were 1-15 % higher than the HPLC results. This may because the extracts contained some oxidizable components other than ascorbic acid. Moreover, the visual titrimetric method was applicable only when the concentration of dehydroascorbic acid was low[38]. So the HPLC technique for ascorbic acid determination in Chinese jujube provided more satisfactory results. However, dehydroascorbic acid contents could be determined by only HPLC since the titrimetric method could just measure ascorbic acid alone.

TABLE-1
COMPARISON OF ASCORBIC ACID AND DEHYDROASCORBIC ACID CONTENTS IN DIFFERENT CULTIVARS OF CHINESE JUJUBE USING DIFFERENT DETERMINATION METHODS

Cultivar	Vitamin C (mg/100 g)			Ascorbic acid / Dehydroascorbic acid
	Ascorbic acid		Dehydroascorbic acid	
	HPLC	Titrimetry	HPLC	
Jinsixiaozao	320.35 ± 3.50c	336.31 ± 8.11d	53.95 ± 3.31d	5.95 ± 0.30b
Zanhuangdazao	369.80 ± 1.95b	374.74 ± 7.80b	60.15 ± 4.60cd	6.17 ± 0.46b
Huizao	225.10 ± 3.12f	248.33 ± 5.55e	65.42 ± 2.85c	3.44 ± 0.11c
Junzao	307.65 ± 2.07d	354.12 ± 4.59c	103.62 ± 8.18a	2.98 ± 0.21c
Pozao	424.74 ± 3.44a	450.50 ± 5.72a	75.67 ± 2.13b	5.64 ± 0.44b
Maoboyan	244.62 ± 5.45e	255.43 ± 4.77e	27.03 ± 2.13e	9.08 ± 0.56a

Each value is expressed as means ± standard deviation of n = 3-6 determinations; Different letters within a column (a-f) denote significant differences ($p < 0.05$) between cultivars.

Analysis of Chinese jujube: The results of ascorbic and dehydroascorbic acid analysis of the six cultivars of Chinese jujube were shown in Table-1. It was very interesting to note the ratio of ascorbic acid and dehydroascorbic acid. Ascorbic acid content of different cultivars varied from 225.10 (Huizao) to 424.74 mg/100 g (Pozao). And the content of dehydroascorbic acid ranged from 27.03 (Maoboyan) to 103.62 mg/100 g (Junzao). Statistical analysis showed ascorbic acid contents of the six cultivars of Chinese jujube exhibited significant difference ($p < 0.05$). The value of ascorbic acid/dehydroascorbic acid varied from 2.98 to 9.08 (Junzao).

It is well know that Chinese jujube is a very good source of ascorbic acid. The results showed that it is also a very good source of dehydroascorbic acid. The dehydroascorbic acid contents in Chinese jujube is 10-100 times more than that in common fruits, such as strawberry, apples, orange, banana, papaya, mango, lemon and so on and 1-10 times more than the common vegetables including green bean, tomato, green pepper and cabbage, in which dehydroascorbic acid is the dominant present form of vitamin C[3,29,30,34].

Conclusion

A simple HPLC method for the determination of two forms of vitamin C (ascorbic acid and dehydroascorbic acid) in Chinese jujube was established, in which dehydroascorbic acid was converted into ascorbic acid before determination using dithiothreitol as reducing agent. It provided more satisfactory results than the conventional titrimetric method. The HPLC method using 0.1 % H_3PO_4 as extraction solution could afford good efficiency of ascorbic acid extraction and stability of ascorbic acid before and after the incubation with dithiothreitol. The determination results of six cultivars showed that Chinese jujube was a very good source of both ascorbic acid and dehydroascorbic acid.

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