Simultaneous Detection of Dehydroascorbic, Ascorbic, and Some Organic Acids in Fruits and Vegetables by HPLC

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A high-performance liquid chromatography method was developed for the simultaneous determination of dehydroascrobic, ascorbic, malic, citric, and oxalic acids in fruits, vegetables, and beverages. Separation of these compounds was accomplished by coupling reversed-phase and organic acid columns using 2% KH_2PO_4 (pH 2.3) as mobile phase with a flow rate of 0.4 mL/min. Detection was performed at 215 and 260 nm using a diode array detector interfaced with portable integrators and a chromatography data system. Selected fresh fruits, vegetables, and commercial orange juices were analyzed using this method.

INTRODUCTION

The ascorbic acid (AA) content of fruits and vegetables can be obtained by titration and colorimetric methods. The visual titration method is based on the reduction of the dye 2,6-dichlorophenolindophenol by the acid solution of AA (AOAC, 1980). At the end point, excess unreduced dye is rose pink in acid solution. This method is applicable to the determination of reduced AA, but in samples where ferrous, stannous, cuprous, sulfite, and thiosulfate ions or SO_2 are present, the method is not applicable. Also in colored samples, detection of the end point is difficult, thereby producing inaccurate results. The colorimetry method uses 2,4-dinitrophenylhydrazine (Roe and Kuether, 1943). It gives an estimation of the total AA after oxidation. However, it is very lengthy, requiring more than 3 h to complete the determination. The spectrophotometric method as modified by the Canada Department of Agriculture (Pearson, 1976) is also based on the oxidation–reduction of AA and the dye 2,6-dichlorophenolindophenol. The method is simple, requiring only about 10 min to determine duplicate samples. As with the previously mentioned methods, however, the results represent AA, giving no quantitative information on dehydroascorbic acid (DHAA). Since L-ascorbic and L-dehydroascorbic acids are the two biologically active forms of vitamin C, it is therefore necessary to quantify these two compounds.

The separation of AA and DHAA by high-performance liquid chromatography (HPLC) has received great attention during the past years. Dennison et al. (1981) employed dual analyses whereby the level of DHAA is obtained by difference. Finley and Duang (1981) separated AA and DHAA using two reversed-phase columns in series with detection at 254 and 210 nm, respectively. A similar detection system was used by Rose and Nahrwold (1981) and Wimalasiri and Wills (1983) for various biological preparations using a single ion-exchange column. Bradbury and Singh (1986) described a separation procedure using a Bondapak-NH_2 Z-module cartridge with the peaks monitored at 210 and 254 nm, respectively. In an attempt to separate AA and DHAA in some fruit and vegetable extracts using a reversed-phase column, it was noted that oxalic acid coeluted with DHAA, thereby giving erroneous results. However, coupling the reversed-phase column with an organic acid column resulted in a better separation of DHAA and oxalic acid, while allowing for detection of other organic acids that are inherently present in fruits and vegetables.

This study describes a chromatographic procedure for the separation of DHAA and AA with the added advantage of detecting other organic acids by coupling reversed-phase and organic acid columns.

MATERIALS AND METHODS

Reagents. A mixture of dehydroascorbic, ascorbic, oxalic, malic, succinic, and citric acids in concentrations of 0.002, 0.02, 0.003, 0.3, 0.1, and 0.5%, respectively, in water was used as standard. Dehydroascorbic acid was prepared by quantitative oxidation of ascorbic acid solution by the addition of bromine until a slight yellow color remained. Excess bromine was displaced by continuously bubbling N_2 through the solution. There was about 95% conversion of ascorbic into dehydroascorbic as shown by HPLC. The mobile phase, prepared using double-distilled water, was filtered through a 0.45-μm Millipore filter and degassed prior to use.

Samples and Sample Preparation. Fresh juice samples were hand-extracted from Hamlin, navel, and Valencia oranges [*Citrus sinensis* (L.) Osbeck], sour oranges (*Citrus aurantium*), grapefruit (*Citrus paradisi* Macf.), lemon [*Citrus limon* (L.) Burm.], and calamondin (*Citrus mitis* Blanco). Valencia oranges were stored at 15.5 °C for 2 months. Commercial processed orange juice samples in open containers were stored for 0, 3, or 6 days at 7 °C. Fruits and vegetables such as carambola, mango, papaya, sweet pepper, and tomato were washed, peeled if necessary, and chopped into small pieces for analysis. Sour oranges and calamondin were obtained from a local arboretum, while the rest were either obtained from packinghouses or purchased from local markets. Juice (50 mL) or edible portions of fruits and vegetables (40–50 g) were blended with 0.05 N H_3PO_4 (50 mL) for 3 min. Solutions of AA in H_3PO_4 were found to be stable for 3 h at room temperature. The volume or weight of the sample has been predetermined in preliminary tests to determine if the peaks were sufficient for quantification. Other acids were also tested to stabilize ascorbic acid during the extraction procedure; however, metaphosphoric, citric, and acetic acids were found to interfere with the analyses. The slurry was centrifuged, and the supernatant was collected and made to 100 mL with the extracting solvent to give the extract. The extract was purified by passing 3 mL through a disposable C_{18} Sep-Pak cartridge (Waters Associates), preconditioned by flushing with acetonitrile (2 mL) followed by double-distilled water (5 mL), and a 0.45-μm Millipore filter prior to injection.

High-Performance Liquid Chromatography. The chromatographic equipment consisted of a solvent-delivery system (Perkin-Elmer isocratic LC pump 250) and a sample injector.

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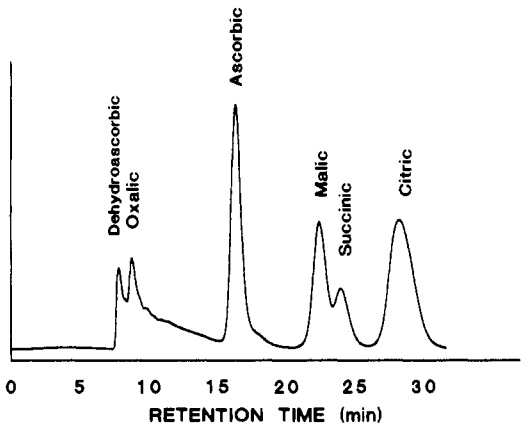

Figure 1. Retention times of standard acid solutions as detected by coupling Spheri-5 RP-18 and Polypore H columns with 2% KH_2PO_4 (pH 2.3) as mobile phase at a flow rate of 0.4 mL/min.

Detection of the acids was performed at 215 and 260 nm using a diode array detector (Perkin-Elmer LC 235) interfaced with two portable integrators (Hewlett-Packard 3396A) and a chromatography data system (Chrom Perfect 2, Justice Innovations). The detector was set at high sensitivity to allow for the absorption of the components that are present in very low levels. Although AA has a maximum absorbance at 245 nm, detection was performed at 260 nm to avoid interference (Finley and Duang, 1981). Because of absorption by interfering compounds at 215 nm, DHAA was quantified by obtaining the difference in absorbance between 215 and 260 nm. The principle of differential spectrophotometry is frequently utilized in clinical studies for quantification in the presence of interfering compounds (Kalckar, 1946). A Spheri-5 RP-18 (110 × 4.6 mm) column and two Polypore H columns (110 × 4.6 and 220 × 4.6 mm) purchased from Brownlee Labs, Inc. (Santa Clara, CA), were used for the separation. The columns were equilibrated with the mobile phase (2% KH_2PO_4, pH 2.3), after which the standard mixture or sample was introduced into the column. The flow rate used was 0.4 mL/min with an accompanying pressure of 670–700 psi.

The levels of the different components in fruit and vegetable samples were determined by comparing the peak areas with those of standards.

RESULTS AND DISCUSSION

Chromatographic Separation of DHAA, AA, and Other Organic Acids. The result for the chromatographic separation of the standard solution by coupled reversed-phase and organic acid columns is presented in Figure 1. Optimal retention of dehydroascorbic, oxalic, ascorbic, malic, succinic, and citric acids was accomplished using these two columns at a mobile phase flow rate of 0.4 mL/min as compared to using either a reversed-phase column alone (Figure 2) or organic acid columns alone (Figure 3). With the reversed-phase column alone, separation was fast but DHAA and oxalic acid coeluted at about 3 min. Separation with the organic acid column was longer, but this time, AA coeluted with malic acid at about 13 min. The elution pattern of the columns connected in series differed from that of the individual columns, as revealed by spectral analyses.

A sample of the chromatographic separation of the different acids obtained from one of the samples (commercial orange juice) is shown in Figure 4. These peaks were identified by spectral analysis, comparison of retention times with those of a standard acid mixture, and spiking the sample with standard components. Results for citrus fruit, commercial orange juice (OJ) samples, and other fruits or vegetables are given in Table I. For fresh citrus fruit samples, DHAA ranged from 0 to 3.9 mg/100 g, AA from 29.1 to 63.4 mg/100 g, malic acid from 0.1 to 0.2%, and citric acid from 0.8 to 3.6%. A 0.1% level of

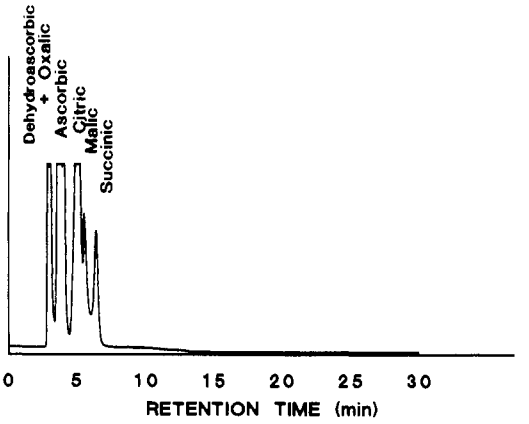

Figure 2. Elution profile of standard acid solutions by Spheri-5 RP-18 with 2% KH_2PO_4 (pH 2.3) as mobile phase at a flow rate of 0.4 mL/min.

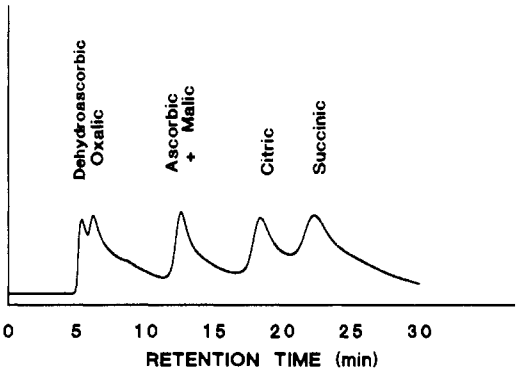

Figure 3. Elution profile of standard acid solutions by Polypore columns with 2% KH_2PO_4 (pH 2.3) as mobile phase at a flow rate of 0.4 mL/min.

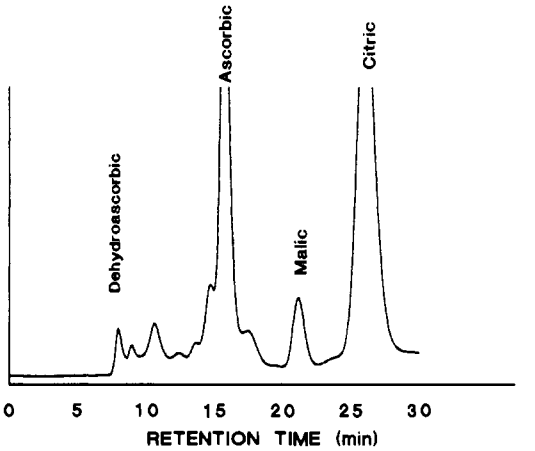

Figure 4. Chromatographic profile of commercial orange juice as determined by coupled Spheri-5 RP-18 and Polypore H columns with 2% KH_2PO_4 (pH 2.3) as mobile phase at a flow rate of 0.4 mL/min.

oxalic acid was detected for carambola, while for mango, tomato, and green pepper, trace amounts were noted. Most of these values are close to or within the range given by Wills et al. (1984), Wimalasiri and Wills (1983), Kefford and Chandler (1970), and Schmandke and Guerra (1969). The levels of AA in whole fruit did not vary appreciably during the 2-month storage period at 15.5 °C. Weight loss probably occurred, but only concentrations of the different components were measured. This observation supports earlier findings that the antiscorbutic factor in whole citrus fruit was well retained during storage, causing slight or no losses of AA in fruit stored at various temperatures for up to 12 weeks (Nagy, 1980).

Table I. Dehydroascorbic, Ascorbic, and Some Major Organic Acids in Selected Fruits and Vegetables

sample	DHAA, mg/100 g	AA, mg/100 g	malic acid, %	citric acid, %	oxalic acid, %
A. fresh citrus fruit					
Tunis sour orange (*C. aurantium* hybrid, North Africa)	3.9	37.9	0.1	2.4	
C. taiwanica (*C. aurantium*, Taiwan)	2.1	29.1	0.1	3.3	
navel orange	2.6	54.1	0.2	1.0	
Hamlin orange	0	52.2	0.2	0.8	
grapefruit	1.2	39.7	tr[a]	1.0	
calamondin	2.2	44.5	0.2	3.6	
lemon	2.0	41.7	0.2	3.5	
Valencia orange					
freshly picked	0.5	60.6	0.1	1.0	
1 week (15.5 °C)	0.2	57.8	0.1	1.2	
2 weeks (15.5 °C)	0.3	63.4	0.1	1.1	
2 months (15.5 °C)	0.4	61.9	0.1	1.2	
B. commercial orange juice (OJ)					
canned OJ (from concentrate)					
0 day	0.1	48.0	0.3	1.3	
6 days	1.6	47.3	0.2	1.3	
reconstituted OJ					
0 day	0	49.8	0.2	1.0	
6 days	0	43.9	0.2	0.9	
aseptically packaged OJ (from concentrate)					
0 day	0	34.1	0.1	0.9	
6 days	1.3	20.4	0.1	0.8	
fresh squeezed OJ					
0 day	2.0	23.4	0.1	1.0	
3 days	2.2	19.3	0.1	1.0	
C. other fruits/vegetables					
carambola (mature green)	4.6	24.0	0.1	tr	0.1
mango, ripe	0.6	27.2	tr	0.2	tr
papaya, ripe	2.6	62.3	tr	0.1	
tomato, ripe	0	26.6	0.1	0.5	tr
green pepper	12.0	71.0	0.1		tr

[a] trace

Table IB reflects the AA values of some commercial juice samples. Higher levels were obtained in OJ samples that were reconstituted from concentrates compared to that of the fresh squeezed sample. Some of the samples showed decreases in AA levels during storage; however, such decreases did not correspond to increases in the DHAA levels. The degradation of AA may have proceeded to products other than DHAA, but their detection was not possible in this procedure.

For other fruits (Table IC), the values obtained by this method agree with those given by Baldwin et al. (1991), Nagy and Shaw (1990), Wills et al. (1984), Wimalasiri and Wills (1983), Davies and Hobson (1981), Schmandke and Guerra (1969), and Lopez et al. (1956).

Reproducibility, Reliability, and Sensitivity. The reproducibility of the chromatographic separation of some of the components was determined by making six injections of the grapefruit sample with the mean and standard deviation (SD) computed for each component. The results are as follows: DHAA, 1.2 ± 0.1 mg/100 g; AA, 39.7 ± 0.6 mg/100 g; and citric acid, 1.0 ± 0.0%. For oxalic acid, six injections of a carambola sample gave this result: oxalic acid, 0.1 ± 0.0%; and malic acid, 0.1 ± 0.0%.

Percent recovery was determined by adding known levels of the acid standard to a juice sample and treating the juice in a manner similar to those of other samples. Recoveries for the different components are as follows: DHAA, 97.5%; AA, 91.3%; oxalic acid, 100%; malic acid, 91.3%; succinic acid, 100.0%; and citric acid, 99.4%. These results indicate that the method has satisfactory accuracy. The method described here can easily detect ascorbic, oxalic, malic, succinic, and citric acids. The detection of DHAA requires a more complicated procedure because of its extremely low UV absorptivity and coelution of interfering substances. These problems were corrected by setting the sensitivity of the detector to an optimum level and by correcting for the area contributed by the interfering substances with the aid of an added integrator.

LITERATURE CITED

AOAC. *Official Methods of Analysis*; AOAC: Washington, DC, 1980; p 746.

Baldwin, E. A.; Nisperos-Carriedo, M. O.; Moshonas, M. G. Quantitative analysis of flavor and other volatiles and for certain constituents of two tomato cultivars during ripening. *J. Am. Soc. Hortic. Sci.* **1991**, *116* (2), 265–269.

Bradbury, J. H.; Singh, U. Ascorbic acid and dehydroascorbic acid content of tropical root crops from the South Pacific. *J. Food Sci.* **1986**, *51* (4), 975–987.

Davies, J. N.; Hobson, G. E. The constituents of tomato fruit—the influence of environment, nutrition and genotype. *CRC Crit. Rev. Food Sci. Nutr.* **1981**, *15* (3), 204–280.

Dennison, D. B.; Brawley, T. G.; Hunter, G. L. K. Rapid high-performance liquid chromatographic determination of ascorbic acid and combined ascorbic acid-dehydroascorbic acid in beverages. *J. Agric. Food Chem.* **1981**, *29*, 927–929.

Finley, J. W.; Duang, E. Resolution of ascorbic, dehydroascorbic and diketogulonic acids by paired-ion reversed-phase chromatography. *J. Chromatogr.* **1981**, *207*, 449–453.

Kalckar, H. M. Differential spectrophotometry of purine compounds by means of specific enzymes. *J. Biol. Chem.* **1946**, *167*, 429.

Kefford, J. F.; Chandler, B. V. *The Chemical Constituents of Citrus Fruits*; Academic Press: New York, 1970; 246 pp.

Lopez, H.; Cimadevilla, M.; Fernandez, E.; Navia, J. H.; Valiente, A.; Clement, I. D.; Harris, R. S. *Bol. Col. Med. Habana*, **1956**, *7*, (10), 334.

Nagy, S. Vitamin C contents of citrus fruit and their products: A review. *J. Agric. Food Chem.* **1980**, *28*, 8–18.

Nagy, S.; Shaw, P. E. *Tropical and Subtropical Fruits*; AVI: Westport, CT, 1990; 570 pp.

Pearson, D. *The Chemical Analysis of Foods*; Churchill: London, 1976; pp 160–161.

Roe, J. H.; Kuether, C. A. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. *J. Biol. Chem.* **1943**, *147*, 399.

Rose, R. C.; Nahrwold, D. L. Quantitative analysis of ascorbic acid and dehydroascorbic acid by HPLC. *Ann. Biochem.* **1981**, *114*, 140–145.

Schmandke, H.; Guerra, O. O. *Nahrung* **1969**, *13* (6), 523–530.

Wills, R. B. H.; Wimalasiri, P.; Greenfield, H. Dehydroascorbic acid levels in fresh fruit and vegetables in relation to total vitamin C activity. *J. Agric. Food Chem.* **1984**, *32*, 836–838.

Wimalasiri, P.; Wills, R. B. H. Simultaneous analysis of ascorbic acid and dehydroascorbic acid in fruits and vegetables by high-performance liquid chromatography. *J. Chromatogr.* **1983**, *256*, 368.

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