HPLC Method for Separation and Determination of Nonvolatile Organic Acids in Orange Juice

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A recently introduced hydrophilic end-capped C_{18} column (YMC ODS-AQ) was tested for the analysis of the nonvolatile organic acids in orange juices. Ten carboxylic acids can be detected from Valencia orange juice by isocratic elution with 20 mM KH_2PO_4 as the mobile phase with a flow rate of 0.7 mL/min and UV detection at 214 nm. Juice samples are purified through a disposable SCX (benzenesulfonylpropyl) extraction cartridge.

INTRODUCTION

The nature and concentration of organic acids in fruits have been of interest because of their important influence on the organoleptic properties of fruit juices. Since each fruit has a unique pattern of organic acids, chromatographic analysis of organic acid could be applied for the verification of juice authenticity (Wrolstad, 1981) and for the estimation of each fruit content from blended fruit juice beverages (Johnson et al., 1992). Citrus fruits are particularly rich in organic acids, the acidic properties of organic acid being used with sugar contents as the chief index of maturity and one of the major analytical measures of flavor quality (Fellers, 1991). Separation and quantitation of organic acids have been considered to be difficult due to their structural similarities and their lack of distinctive spectral properties (Keefer and Schuster, 1986). Furthermore, most of the organic acids of interest in fruit juices are weak acids with similar pK_a values. They cannot be separated quantitatively if one of the acids is present in relatively large amounts.

Several HPLC methods for the determination of organic acids in citrus juice products have been developed in ion-exchange chromatography (Lee and Lord, 1987; Turkelson and Richards, 1978) and reversed-phase chromatography with C_{18} bonded-phase columns (Badoud and Pratz, 1986; Keefer and Schuster, 1986; Mentasti et al., 1985; Nisperos-Carriedo et al., 1992; Lee et al., 1990; Shaw and Wilson, 1983; Shaw et al., 1983). Reversed-phase HPLC utilizing buffered eluents has been more attractive for the analysis of organic acids but generally did not provide good separations for all of the acids of interest. For better results, coupling of two columns in series (Lee et al., 1990; Nisperos-Carriedo et al., 1992) or gradient elution with precolumn derivatization (Badoud and Pratz, 1986; Mentasti et al., 1985) and addition of ion-pairing agents (Keefer and Schuster, 1986) was needed.

This paper describes an improved reversed-phase HPLC procedure with a hydrophilic end-capped C_{18} column for the determination of carboxylic acids in orange juice.

EXPERIMENTAL PROCEDURES

Chemicals and Supplies. Carboxylic acids (or their sodium salts) were purchased from Sigma Chemical Co. (St. Louis, MO) and Fisher Scientific Co. Valencia orange [*Citrus sinensis* (L). Osbeck] juice samples were obtained from the pilot plant at CREC-University of Florida, Lake Alfred, FL. The SCX (benzenesulfonylpropyl) extraction cartridge (100 mg) was obtained from Varian (Harbor City, CA). HPLC ultrapure water was generated through a Milli-Q water purification system (Millipore, Bedford, MA). Disposable filters (0.45 μm, Acro LC13) were purchased from Gelman Sciences (Ann Arbor, MI).

Standard Solutions. Stock solutions of the standards were prepared using free acids (except succinic acid, which was a disodium salt) at a concentration of 1.0 g/100 mL in water. Propionic acid in water was used as the stock internal standard solution. The working standard mixtures were prepared by mixing equal volumes of the stock solutions, and internal standard with water, to make 0.1 g/100 mL of each compound after dilution except for *cis*-aconitic, fumaric, lactic, and shikimic acids. The working standards of *cis*-aconitic, fumaric, lactic, and shikimic acids, were prepared at a concentration of 0.001 g/100 mL.

Chromatographic Equipment. The chromatographic system consisted of a Waters Model 600E system controller/pump (Waters Associates, Inc., Milford, MA), a Rheodyne Model 7725i injector, a Spectra-Physics Model 200 programmable wavelength UV detector, and a YMC-Pack ODS-AQ column with 5-μm packing (AQ-303, 120 Å, 250 × 4.6 mm i.d.). The column was thermostated to 25 °C in a Waters column temperature control module. Integrations and data storage were performed with APEX chromatography software (Autochrom, Inc., Milford, MA) with the aid of a 486 AT-style computer. On-line spectral acquisition was performed by connecting the Waters 990+ photodiode array detector in series.

Analytical Condition. Analysis of organic acids was carried out by injecting 20-μL aliquots of sample or standards onto the column. The organic acids were eluted isocratically with 20 mM KH_2PO_4 (pH 2.8) at a flow rate of 0.7 mL/min. The eluate was monitored at 214 nm.

Sample Preparation. A sample of juice (8 mL) was mixed with 1 mL of 2.5% metaphosphoric acid and 1 mL of 2% propionic acid and centrifuged for 5 min at 5000 rpm. A SCX sample preparation cartridge was pretreated with 1 mL of methanol and rinsed with 10 mL of distilled water before use. A 1-mL aliquot of centrifugate was pipetted and passed through the pretreated SCX cartridge and collected into a 13-mL centrifuge tube. The cartridge was washed with 2 mL of distilled water and collected into the centrifuge tube with the treated sample, and the volume was adjusted to 4 mL with mobile phase. Sample was filtered through a 0.45-μm filter prior to injection.

Recovery Studies. Percent recovery was accomplished by spiking the known levels of organic acids of interest (malic, ascorbic, citric, fumaric, succinic, and *cis*-aconitic) into orange juice and subjecting the mixture to SCX cartridge treatment. Six injections each of duplicate preparations of the two different juice samples were made and averaged.

RESULTS AND DISCUSSION

The effect of eluent pH on capacity factor (K') of organic acids which have been reported in citrus fruits is demonstrated in Figure 1. The reported pK_a values of these acids (Dean, 1992; Lee and Lord, 1987; Ulrich, 1971) are also presented in Table I. The pH can be a powerful factor

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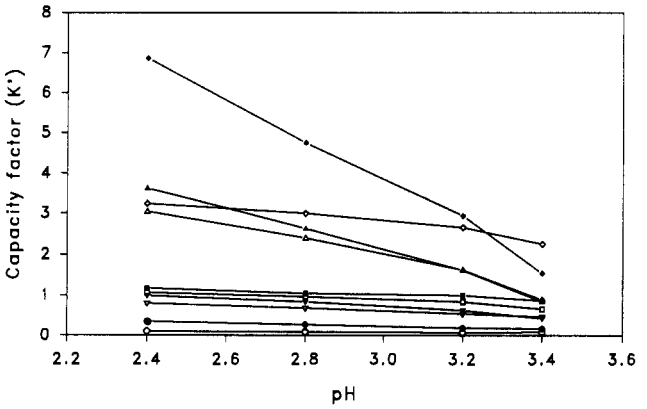

Figure 1. Effects of pH on the capacity factor (K') of organic acids. Other parameters are identical to those of Figure 2. Oxalic (○), tartaric (●), malic (▽), isocitric (▼), lactic (□), ascorbic (■), citric (△), fumaric (▲), succinic (◇), and *cis*-aconitic acid (◆).

Table I. Effects of Ionic Strength on the Capacity Factor (K') of Organic Acids and Response Factor (RF) of Each Acid

		capacity factor (K')				
acid	pK_a	10 mM	20 mM	30 mM	40 mM	RF[a]
oxalic	1.2, 4.2	0.09	0.09	0.07	0.07	6.3
tartaric	2.9, 4.3	0.28	0.27	0.26	0.25	1.5
quinic		0.34	0.34	0.32	0.32	0.5
malic	3.4, 5.2	0.71	0.68	0.67	0.66	0.7
malonic	2.8, 5.7	0.75	0.74	0.71	0.71	0.8
isocitric		0.78	0.77	0.76	0.76	0.5
α-ketoglutaric	2.6	0.98	0.95	0.93	0.92	7.9
lactic	3.9	1.01	0.98	0.95	0.95	1.3
ascorbic	4.2, 11.6	1.08	1.07	1.05	1.03	14.3
citric	3.1, 4.8, 6.4	2.26	2.23	2.17	2.15	0.4
fumaric	3.1, 4.6	2.57	2.57	2.52	2.51	126.9
succinic	4.2, 5.6	2.91	2.86	2.82	2.75	0.2
propionic[b]	4.8	4.32	4.25	4.16	4.09	1
cis-aconitic	2.8, 4.5	4.51	4.46	4.32	4.32	58.6

[a] $RF_x = (A_x \times W_{is})/(A_{is} \times W_x)$, where A_x and A_{is} are peak area of sample and internal standard and W_x and W_{is} are concentration (mg/100 mL) of sample and internal standard, respectively. [b] Included as an internal standard.

in the separation of nonvolatile organic acids which can be ionized or protonated according to the pH of the condition. The average pK_1 value of the organic acids in Table I was about 3.1. To compare the band spacing and retention, the pH of the aqueous solution of the mobile phase was varied near the average of pK_1 values of the acid mixtures (2.4, 2.8, 3.2, and 3.4) with phosphoric acid while the other parameters were kept constant.

As the eluent pH is increased, these acids become more ionized and the K' declines. Oxalic acid seems to be completely ionized under tested pHs of the mobile phase; its retention was at a minimum (Table I). Most organic acids are too weak to be affected by pH changes in the 2.4–3.4 range. However, retention of moderately strong organic acids, such as fumaric, citric, and *cis*-aconitic acids, shows a definite pH dependence. The peak of citric acid, which is known to be predominant in citrus, could not be resolved from ascorbic acid at pH 3.4. The fumaric acid peak could not be resolved from citric acid at pH 3.2. Also, selectivity between fumaric and succinic acids changed between pH 2.4 and 2.8 (Figure 1). Band spacing is better at pH 2.4 than at pH 2.8 due to more protonation of the acids. However, pH 2.8 was preferred because of its shorter analysis time and subsequently prolonged the column life.

Table I shows the effect of buffer strength on the separation of organic acids which have been reported in

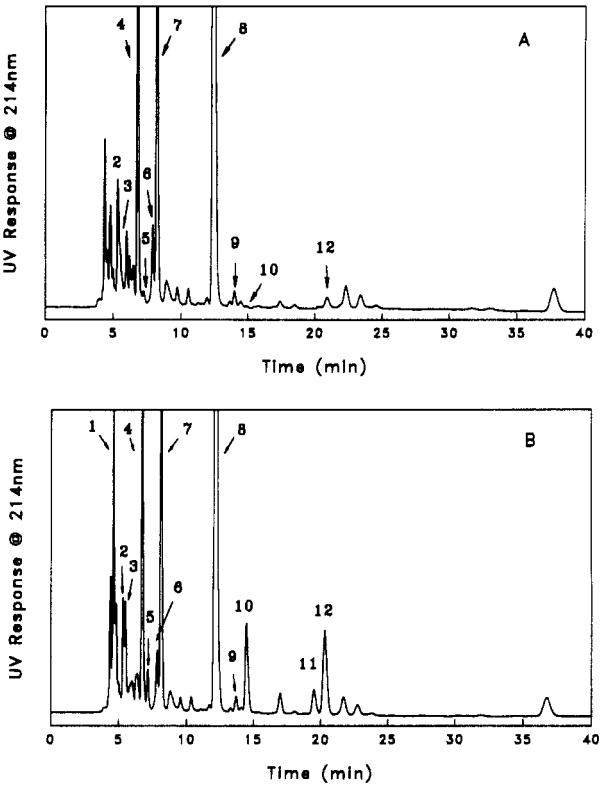

Figure 2. Separation of organic acids in (A) orange juice and (B) orange juice spiked with some of the standard acids. Column, 25 cm × 4.6 mm YMC ODS-AQ; mobile phase, 20 mM KH_2PO_4 (pH 2.8); flow rate, 0.7 mL/min; detection, UV at 214 nm. Peaks: 1, oxalic; 2, quinic; 3, tartaric; 4, malic; 5, isocitric; 6, unknown; 7, ascorbic; 8, citric; 9, fumaric; 10, succinic; 11, propionic; and 12, *cis*-aconitic acid.

citrus fruits. The pH was maintained at 2.8. In this mixture, the acids are protonated at the pH of the mobile phase and probably interact with the column packing silanols by ion exchange. Increasing the salt concentration does not change the selectivity for these acids but seems to cause decreases in ion-exchange interaction and decreases in the retention of these acidic compounds. Seven of 14 tested organic acids fall within K' values of 1–10, which is suggested as the desired range for multicomponent analysis (Snyder and Kirkland, 1979); this number decreased to 6 as the buffer concentration increased to greater than 20 mM (Table I). However, it has been suggested that chromatography with less than 20 mM buffer concentration can be overly sensitive to small changes in buffer concentration (Dolan, 1992). A 20 mM buffer concentration was chosen as optimum buffer strength for this study since it provided good resolution for these acids, and further increases up to 40 mM did not give any significant gains in resolution of these acids.

Figure 2A shows the separation of the organic acids present in orange juice. Each acid was identified by its retention time in comparison with standard solutions of pure acids and based on spiking of the standards. Figure 2B clearly shows the separation of 11 acids by spiking the known standards of some of the minor acids such as oxalic, quinic, isocitric, succinic, and *cis*-aconitic acids into the orange juice. Propionic acid was also spiked as an internal standard to facilitate identification of acids, and metaphosphoric acid was added to help stabilize ascorbic acid during analysis. As seen from Figure 2A, the major nonvolatile organic acids in orange juice are citric and malic, but a variety of other acids such as oxalic, quinic, tartaric, isocitric, ascorbic, fumaric, succinic, and *cis*-aconitic are present in small amounts. Even though,

fumaric and *cis*-aconitic acids were present at very low concentrations (0.4 mg/100 mL for fumaric acid and 0.3 mg/100 mL for *cis*-aconitic acid), they could be detected easily because of their high extinction coefficients (Keefer and Schuster, 1986; Turkelson and Richards, 1978). The detection limits of fumaric and *cis*-aconitic acids were about 10 and 20 μg/100 mL, respectively, with a signal-to-noise ratio of 4 under this condition. Succinic acid appears in more than trace amounts (46.7 mg/100 mL) in this orange juice, but it seems to be difficult to quantitate due to its relatively low response factor compared to those of other acids. The response factor for succinic acid was about 0.2, the lowest value among the tested organic acids for this study. The response factor of each acid, which is calculated on the basis of relative response to internal standard of propionic acid, is also presented in Table I. Peak 6 has the same retention time as shikimic acid, but spectral analysis cannot confirm the peak. Shikimic acid is common to fruit juices such as apple, pear, strawberry, and banana (Ulrich, 1971), which is not surprising because it is a precursor for quinic acid and aromatic amino acids, but its presence in citrus juice products has only been reported in a recent work with commercial orange juice products by HPLC and isotachophoresis (Busturiusa, 1992).

In the sample preparation, SCX sorbent, which has a functional group of benzenesulfonic acid, was used to purify the juice samples. Numerous aromatic compounds and cations from the juice matrix could be removed by the mixed mode of hydrophobic and ion-exchange capacity of SCX sorbent treatment as described in a previous work (Senden et al., 1992). Most of the organic acids of interest were not retained, and more than 95% of spiked amounts were recovered from SCX sorbent except citric and *cis*-aconitic acids. HPLC analysis showed 92.9% (RSD = 2%) recovery of citric acid and 93.1% (RSD = 2.8%) recovery of *cis*-aconitic acid, respectively.

Reproducibility of the HPLC method, which was estimated by duplicate analysis of six juice samples prepared from the same orange juice on the same day, was between 0.1 and 0.4% RSD in migration time for the six organic acids (malic, ascorbic, citric, fumaric, succinic, and *cis*-aconitic acids) within the same day and between 1.1 and 6.4% RSD variability in quantitation of these six acids. Flushing the column with 30 mL of water followed by 30 mL of 50% aqueous acetonitrile after daily work seems to maintain the column integrity.

On-line spectra of acids indicated that there were no distinctive spectral properties in aliphatic carboxylic acids as described earlier (Keefer and Schuster, 1986) except for the spectra of fumaric and *cis*-aconitic acids. The spectra of fumaric acid (λ_{max} = 216 nm) and *cis*-aconitic acid (λ_{max} = 220 nm) are similar to the spectrum of a carbocyclic monocarboxylic acid such as shikimic acid, which has maximum absorption at 218 nm. The spectrum of ascorbic acid also could easily be distinguished from the spectra of aliphatic carboxylic acids due to its maximum absorption at 252 nm in this mobile phase.

In conclusion, the results of the present study suggest that RP-HPLC with a single reversed-phase column of a relatively hydrophilic surface (ODS-AQ) offers good separation prospects for the determination of nonvolatile organic acids in orange juice. This method could be applied to other noncitrus fruit juices. HPLC analysis time was less than 40 min, but the major advantage over conventionally end-capped ODS packing is the capability to retain the polar carboxylic acids longer and provide improved resolution.

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