High-Performance Liquid Chromatographic Separation of Carboxylic Acids with Anion-Exchange and Reverse-Phase Columns

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Conditions were examined for separation of various carboxylic acids using various reverse-phase, silica-based weak anion-exchange, and polymeric strong anion-exchange columns. Column effluents were monitored with UV or RI detectors, depending on the method of elution. For the separation of tricarboxylic acid cycle and related acids, the choice of column depends on the complexity of the sample, as well as the quantitative distribution of individual acids. Optimal eluting conditions depend on the distribution of acids.

Various methods have been employed for the liquid column chromatographic separation of organic acids (Jandera and Churacek, 1973). Primary difficulties in the separation and detection of acids have been due to the broad range of polarities of the acids (normally overcome by gradient elution) and the low detector sensitivity for underivatized carboxylates. Modern refractive index (RI) detectors can partially compensate for low sensitivity, if isocratic conditions can be used and the solvent has an RI sufficiently different from the sample ions. On the other hand, detection by ultraviolet absorption (UV) at low wavelengths (206–214 nm) is often limited by the absorbance of the eluent. If halide salts are excluded, this limits the available eluents to solutions of a few inorganic salts (Buck et al., 1954).

In this report, we describe chromatographic separation of various carboxylic acids on (1) reverse-phase columns (C-8) and radial compression columns (C-18 and C-8 functionality) with ion suppression in phosphate buffers, (2) propylamine and diethylaminoethyl (DEAE) functionality weak anion-exchange columns with isocratic phosphate buffers, and (3) a polymeric strong anion-exchange column using sulfate buffers under both gradient and isocratic conditions.

MATERIALS AND METHODS

The chromatography was performed with a Waters Model 202 liquid chromatograph equipped with a Model R401 differential RI detector without thermostating and either a fixed 254-nm UV detector or a variable-wavelength detector (Schoeffel Model 770 or LDC Spectromonitor III). Sample injection was made with either a Rheodyne 7120 injection valve equipped with a 20-μL loop or a Waters U6K injector. For gradient programming a Waters Model 660 programmer and an additional Model 6000A pump were used. Columns used were as follows: (a) 10-cm radial compression columns with C-18 or C-8 functionality, 10 μm (Waters); (b) 25 cm × 4.6 mm i.d. C-8 analytical columns of two manufacturers, 6 and 10 μm (Du Pont and Whatman); (cc) 25 cm × 4.6 mm propylamine column, 6 μm (Du Pont); (d) 25 cm × 4.6 mm DEAE functionality column, 5 μm (Serva-Accurate Chemical and Scientific Co.); (e) 25.5 cm × 5 mm column packed with Hamilton HA-X8.00, 7-10-μm strong anion-exchange resin in the sulfate form.

A Hewlett-Packard Model 3380A integrator was used to record and quantitate the peaks for comparison.

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Standard mixtures of acids, obtained from commercial sources, were prepared in water or in the appropriate buffer. Concentration of the standard acids was 1% when the RI detector was used. For UV detection, concentrations of fumarate and *cis*-aconitate were 1 mM and all other acids 10 mM.

The anion-exchange resin column was packed by attaching the column blank equipped with a 2-μm exit frit to a 40 cm × $^3/_8$ in. tube and adding the suspended packing (sonicated and deaerated under vacuum) as a thick slurry to the tube in 1 M ammonium sulfate solution. The slurry was allowed to settle for 30 min, and the column and the tube were attached to a Waters 6000A pump at a flow rate of 9.5 mL/min. When the packing pressure was stable (about 4000 psi), the pumping was discontinued and the column was fitted with an inlet frit and low dead volume fitting. A guard column (3.5 cm × 3.2 mm) was packed with the same material in a similar manner. The guard column for the DEAE weak anion exchanger was 4 cm × 2 mm and for all other columns 4 cm × 4.6 mm, dry-packed with a larger particle (20 μm) size version of the main analytical column packing.

The mobile phases used were as follows: 2% $NH_4H_2PO_4$ adjusted to pH 2.4 with phosphoric acid for all reverse-phase columns (Shaw and Wilson, 1981); 0.15 M NaH_2PO_4 (pH 4.2) for the propylamine column; 0.30 M $NH_4H_2PO_4$ adjusted to pH 6.5 with concentrated ammonium hydroxide for the DEAE column; 0.5 M $(NH_4)_2SO_4$ adjusted to pH 7.25 with ammonium hydroxide, 0.3 M $(NH_4)_2SO_4$ containing 10% methanol (Mallinckrodt Chromar), and 0.1 and 1.5 M $MgSO_4$ solutions (for the gradient analysis) for the strong anion-exchange columns. The strong anion-exchange column was used at 80 °C, and the rest were used at ambient temperatures unless otherwise noted. Flow rates were as indicated on the chromatograms.

RESULTS AND DISCUSSION

Reverse Phase. Figure 1 shows separation on a variety of HPLC columns for a standard mixture of six organic acids commonly found in fruits. A guard column was attached to each column despite some loss in resolution, because use of a guard column is often necessary in analysis of natural product mixtures to maintain column performance (Shaw and Wilson, 1981). Figure 1a shows separation on a radial compression C-18 column of the standard mixture of six acids. Oxalic and tartaric acids are not resolved under these conditions; thus, natural products containing both acids could not be satisfactorily analyzed. Similarly, fumaric and succinic acids are incompletely resolved (R = 0.67).

In order to more fully evaluate the radial compression C-18 column for analysis of organic acids, we determined the capacity factors for 20 organic acids under identical conditions using both RI and UV (254 nm) detectors.

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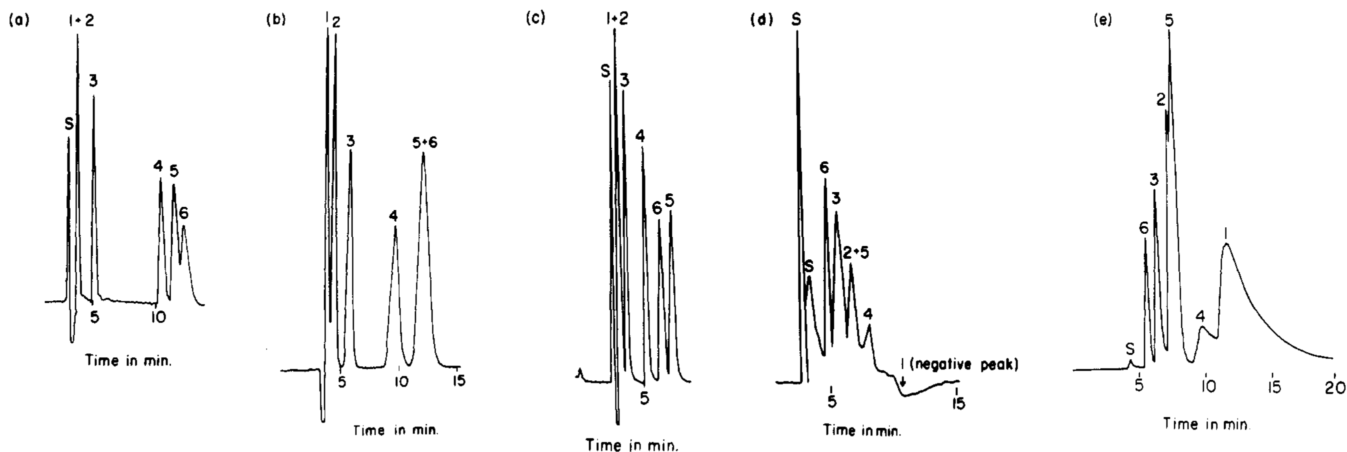

Figure 1. HPLC chromatograms obtained from a mixture of organic acids on columns: (a) 10-cm radial compression C-18; (b) 10-cm radial compression C-8; (c) 25-cm reverse-phase C-8; (d and e) 25-cm bonded phase propylamine column. Sample size: 20 μL (1% w/v each compound). Solvent flow rate: 1.0 mL/min. The refractive index detector was used except the variable-wavelength UV (206 nm) detector was used for (e). For other chromatographic conditions, see Materials and Methods. Peak identities: S, solvent; 1, oxalic acid; 2, tartaric acid; 3, malic acid; 4, citric acid; 5, fumaric acid; 6, succinic acid.

Table I. Retention Order of Acids on RCM-100 C-18 at 1.0 mL/min

acid	K'	UV (254 nm)
oxalic	0.20	yes
galacturonic	0.20	no
tartaric	0.32	no
glycolic	0.36	no
oxaloacetic	0.38	yes
quinic	0.39	yes
tiglic (2-methyl-2-butenoic)	0.42	yes
dehydroascorbic	0.42	yes
formic	0.43	no
pyruvic	0.60	yes
malic	0.71	no
shikimic	0.82	yes
ascorbic	0.94	yes
α-ketoglutaric	1.03	yes
lactic	1.11	no
α-ketobutyric	1.97	yes
citric	2.34	no
fumaric	2.71	yes
succinic	2.98	no
cis-aconitic	2.98	yes

Table I lists the acids chromatographed, their capacity factors (K') by RI detection, and whether or not they had sufficient UV absorption at 254 nm to be detected. Many natural product mixtures contain only a few of the acids listed in Table I and, thus, could probably be analyzed on this column. However, acids that elute near the solvent front, e.g., oxalic, galacturonic, and tartaric, are especially difficult to quantitate because the width of the solvent front may differ between injections. This variation affects detection by RI much more than by UV.

A mixture of six organic acids was separated on a radial compression C-8 column and on two different 25-cm reverse-phase C-8 columns; the results were compared to those from the radial compression C-18 column (Figure 1a–c). The radial compression C-8 column was the only one of these that resolved oxalic and tartaric acids. The retention order for fumaric and succinic acids was reversed on the radial compression C-18 column from that on the reverse-phase C-8 columns. The radial compression C-8 column did not even partially resolve these two acids. Two 25-cm reverse-phase C-8 columns purchased from different sources showed similar separation characteristics except that the column from one source (not shown in Figure 1) failed to completely resolve fumaric and succinic acids (R = 0.43). An additional column from this same source purchased over 1 year later did not resolve the mixture of six acids (a single large peak at 3.50 min) under the conditions described. This observation emphasizes the difficulty often encountered in duplicating separation on columns believed to be identical.

Ion Exchange. Separation of the mixture of six organic acids on a propylamine column gave considerably different results (Figure 1d,e) from those found for C-8 and C-18 columns. The two acids (oxalic and tartaric) that eluted earliest and were not separated on the reverse-phase C-8 and radial compression C-18 columns were well separated on the amine column and were two of the later eluting acids. However, tartaric and fumaric acids were incompletely separated, and oxalic acid had a negative RI relative to that of the buffer. Thus, oxalic acid was impossible to quantitate with the RI detector under the conditions used (Figure 1d). Oxalic acid was readily detected at either 206 nm (Figure 1e) or 254 nm with a UV detector, but a severely tailing peak was obtained for this acid at room temperature by using an aqueous buffer. At 70 °C with 5% methanol added to the buffer, the peak was sharp enough for accurate integration.

Separation of the organic acids commonly found in some foods, including fruit juices, is frequently complicated by the nonresolution of some acids or the predominance of one of the species. The latter may be due to addition of certain acids or natural production by the fruit. Earlier work (Busch et al., 1952; Bengtsson and Samuelson, 1971; Palmer and List, 1973) indicated the usefulness of anion-exchange resins in the separation of a wide variety of acids, especially of the tricarboxylic acid cycle. All these earlier methods have one or more problems which make them relatively unsuitable for rapid HPLC. Specifically, HCl was commonly used as an eluent in earlier liquid column chromatographic methods, but it is unsuitable for use in HPLC instruments because they are so delicate. In addition, the excessive elution times common to the earlier methods make them only marginally useful for routine analyses.

Detection at 206 nm is restricted by the absorbance of numerous inorganic salts (Buck et al., 1954). So that this could be overcome, several sulfates were spectrally analyzed (1 M solutions from 200 to 300 nm) to determine their suitability as eluents. Selected batches of $(NH_4)_2SO_4$, or $(NH_4)_2SO_4$ recrystallized from water which had an absorbance below 0.2A at 206 nm (1 M) and below 0.1A at

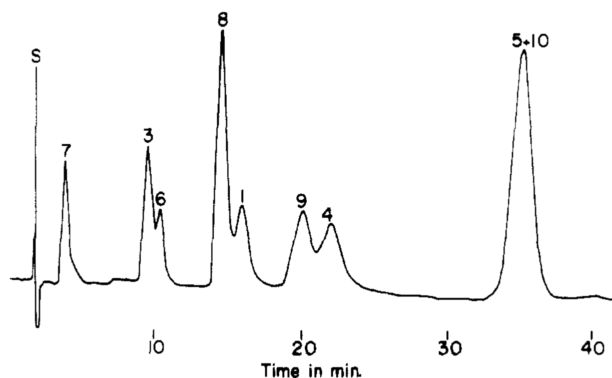

Figure 2. Chromatogram of organic acid mixture on HA-X8.00 anion-exchange resin (7–10 μm). UV detection at 206 nm, 0.08 aufs. Amount injected was 20 nmol of fumaric and *cis*-aconitic acid and all others 200 nmol/injection volume of 20 μL. Mobile phase: 0.5 M ammonium sulfate solution, pH 7.25. Flow rate: 1.5 mL/min. Column temperature: 80 °C. Peak identities: 1–6 as in Figure 1; 7, quinic acid; 8, malonic acid; 9, isocitric acid; 10, *cis*-aconitic acid (*11*, *trans*-aconitic acid).

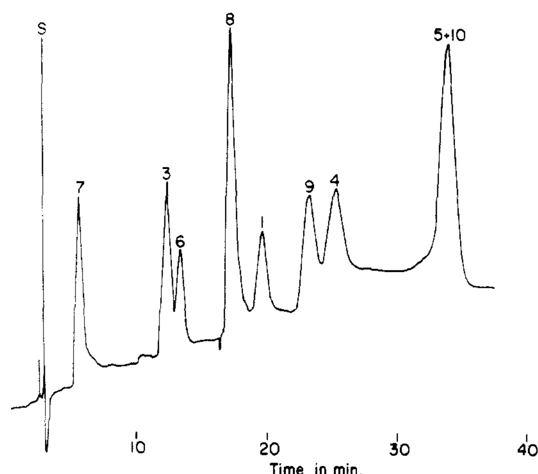

Figure 3. Chromatogram of organic acid mixture on HA-X8.00 anion-exchange resin (7–10 μm). Linear flow program from 1 to 2 mL/min in 30 min. Detection at 206 nm. 0.08 aufs. Other conditions are as in Figure 2.

210 nm, were found suitable, along with reagent-grade $MgSO_4$. Some $(NH_4)_2SO_4$ samples showed unusually high absorbance between 200 and 220 nm, and these samples contained a contaminant that interferred with detection because it eluted from the column as a broad, late-emerging peak. Therefore, it was necessary to screen samples of $(NH_4)_2SO_4$ before use.

Figure 2 shows the separation of nine acids found in fruit juices, in particular citrus juices, on the Hamilton HA-X8.00 resin (7–10-μm particle size) in 0.5 M $(NH_4)_2SO_4$ (pH 7.25). With the exception of fumaric and *cis*-aconitic acids, which do not separate under these conditions, a reasonable separation is achieved. Sensitivity of detection at 206 nm shows marked improvement over that of the RI detector. Nevertheless, when one component predominates, a better separation can be obtained by flow programming (Figure 3) at the expense of some base-line rise. This shows the sensitivity of the Schoeffel Model 770 detector to pressure changes. Some additional improvement was achieved when the concentration of $(NH_4)_2SO_4$ was reduced to 0.3 M and 10% methanol was added to the mobile phase (Figure 4). This system did not separate fumaric and *cis*-aconitic acids; α-ketoglutaric acid also was not separated from fumaric and *cis*-aconitic acid under these conditions. A gradient of $(NH_4)_2SO_4$ improved peak shapes, but a severe rise in the base line resulted.

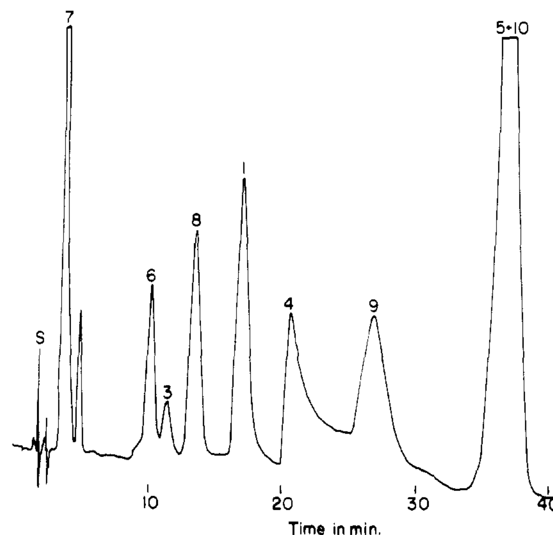

Figure 4. Chromatogram of organic mixture on HA-X8.00 anion-exchange resin (7–10 μm). Mobile phase: 0.3 M ammonium sulfate solution in 10% methanol, pH 7.25. Flow rate: 1.5 mL/min. Column temperature: 80 °C. UV detection at 206 nm. Peak identities are as in earlier chromatograms.

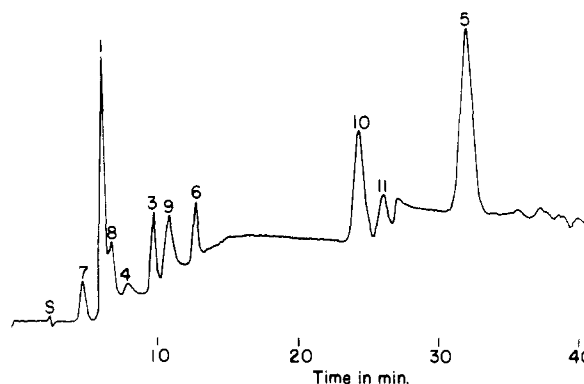

Figure 5. Chromatogram of organic acid mixture on HA-X8.00 anion-exchange resin (7–10 μm). Mobile phase: linear gradient from 0.1 to 1.5 M magnesium sulfate solution in 40 min. Flow rate: 1.5 mL/min. Column temperature: 80 °C. UV detection at 206 nm. 0.16 aufs. Peak identities are as in earlier chromatograms.

Jansen and Samuelson (1971) in their work on hydroxy acids suggested using the formation of a complex with cations for improving separation. We used $MgSO_4$ solutions under isocratic conditions and found the mixture of acids separated poorly. However, since the $MgSO_4$ had relatively low absorbance in the low UV range, a gradient system could be used. Figure 5 shows the separation achieved. Although some rise in base line is unavoidable using a gradient program, it can be minimized by selecting a higher wavelength if reduced sensitivity can be tolerated. Under these conditions, all components separated satisfactorily, including the small amount of *trans*-aconitic acid present in the *cis*-aconitic acid sample. However, the order of separation of components was entirely different from that obtained when the eluent did not contain a complexing cation.

A newer silica-based, weak anion-exchange column (Servachrom Si 100:Polyol:DEAE) with 5-μm particle size was also used to separate mixtures of acids. Figure 6a shows the separation achieved with the same mixture of acids as was used with the polymeric resin. The separation was accomplished in less than 0.5 h by using a relatively low concentration of $NH_4H_2PO_4$ solution, adjusted to pH 6.5. The results also show that elevated temperatures were

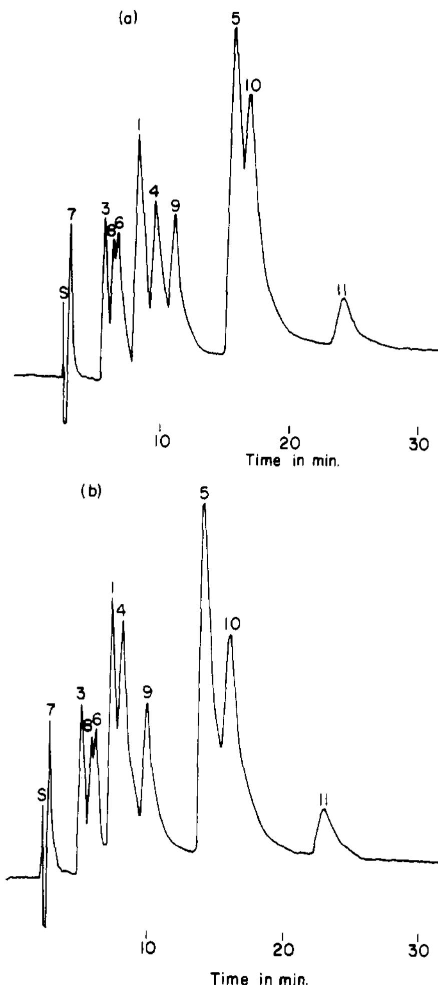

Figure 6. Chromatogram of organic mixture on Servachrom Si 100:Polyol:DEAE weak anion-exchange column. Mobile phase: 0.3 M ammonium dihydrogen phosphate adjusted to pH 6.5 with ammonium hydroxide (a); 0.3 M potassium dihydrogen phosphate adjusted to pH 6.5 with potassium hydroxide (b). Flow rate: 1.0 mL/min. Column temperature: ambient. UV detection at 206 nm. 0.08 aufs. Peak identities are as in earlier chromatograms.

not necessary for separation with this column. As the exchange capacity of this column is much lower than that of the polymeric resin, lower sample loading is required or unexpected retention shifts can occur. However, separation efficiency, as indicated by peak widths, is much better than that with the polymeric exchangers.

We observed a slight change in chromatographic separation when potassium ion was used instead of ammonium ion in the separating buffer solution (Figure 6b). Although separation can be adequate at the pH of the appropriate concentration of dihydrogen phosphate salts (ca. pH 4.5), better separation was achieved at pH 6.5. The peaks, although distinct, were tailing noticeably under both conditions. However, the column performance degrades somewhat over a period of time at the higher pH. To retard this degradation in performance, it is necessary to store the columns overnight in salt-free condition in acetonitrile. The column was washed for 15 min with water at 1 mL/min, followed by 10 min or longer of acetonitrile before shutting the system off. For reequilibration of the system with buffer, water was pumped through the column for 10 min, followed by pH 6.5 buffer for 15 min before the first injection.

CONCLUSIONS

All types of columns tried were capable of separating some of the organic acids found in fruit. other foods containing some of these acids may also be analyzed. The anion-exchange column separates the largest number of acids. However, some acids had relatively long retention times, and temperature elevation (80 °C) was required to obtain sharp symmetrical peaks. Reverse-phase C-8 and C-18 and propylamine columns separated acid mixtures in relatively short times (~15 min) and generally afforded sharp, symmetrical peaks at ambient temperatures. However, many acids were incompletely separated on these columns. The DEAE column gave good separation under conditions tried, but tailing was quite prominent. Thus, choice of column type, column temperature, and elution solvent or solution depends on the precise mixture of acids to be separated, and no one column seems to be best for all acid separations. For routine separations of samples from a variety of sources, preliminary experiments are necessary to establish the nature of the acid mixture for best column selection. So that good analyses with UV detection can be obtained, careful selection of the eluting buffers is necessary.

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