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Ion-Exclusion High-Performance Liquid Chromatography of Aliphatic Organic Acids Using a Surfactant-Modified C18 Column

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Abstract

Ion exclusion chromatography (IELC) of short chain aliphatic carboxylic acids is normally done using a cation exchange column under standard HPLC conditions but not in the ultra-HPLC (UHPLC) mode. A novel IELC method for the separation of this class of carboxylic acids by either HPLC or UHPLC utilizing a C18 column dynamically modified with sodium dodecyl sulfate has been developed. The sample capacity is estimated to be near 10 mM for a 20 µL injection or 0.2 µmol using a 150 × 4.6 mm column. The optimum mobile phase determined for three standard mixtures of organic acids is 1.84 mM sulfuric acid at pH 2.43 and a flow rate of 0.6 mL/min. Under optimized conditions, a HPLC separation of four aliphatic carboxylic acids such as tartaric, malonic, lactic and acetic can be achieved in under 4 min and in <2 min in the UHPLC mode at 2.1 mL/min. A variety of fruit juice and soft drink samples are analyzed. Stability of the column as measured by the retention order of maleic and fumaric acid is estimated to be ~4,000 column volumes using HPLC and 600 by UHPLC. Reproducible chromatograms are achieved over at least a 2-month period. This study shows that the utility of a C18 column can be easily extended when needed to IELC under either standard or UHPLC conditions.

Introduction

Since its introduction in 1953 (1), ion exclusion chromatography (IELC) has been widely used to effectively separate short chain aliphatic acids, primarily carboxylic acids, using inorganic acid eluents (2–4). Separations of short chain aliphatic acids, by IELC, are commonly performed on strong cation exchange columns, in which the negatively charged sulfonate groups form a shield around the stationary phase, called the Donnan membrane. The Donnan membrane repels negatively charged analyte ions, but allows neutral or partially ionized species to penetrate the membrane and interact with the underlying resin (5–7). For this reason, hydrophobic adsorption must be considered as part of the retention mechanism in addition to exclusion (8). Therefore, IELC should be thought of as having a mixed retention mechanism (9). In general, carboxylic acids with higher pK_a values and more hydrophobic sites show longer retention, due to less repulsion and more hydrophobic adsorption.

Determination of aliphatic organic acids is important to the food industry. Such acids play a significant role in influencing flavor, appearance and smell of foods and beverages. Determination of the type and concentration of organic acids is important to ensure the quality of the food, especially fruit juices, wines and beverages. Analytical methods for the determination of carboxylic acids include enzymatic analysis, gas chromatography (10), capillary electrophoresis (11, 12) and liquid chromatography. Many of these methods involve time-consuming sample preparation and lengthy separations involving organic solvent mobile phases and are therefore inadequate for rapid, sensitive and reliable determination of acid analytes in foods and beverages (13).

The separation of short chain aliphatic acids has been performed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a variety of column stationary phases. Early work in this field explored silica-based C18 columns for such a separation.

Most separations were performed in <40 min, with a combination of organic and inorganic eluents with gradient elution (14–17). The development of polymeric RP resin columns allowed for greater stability over a wider pH range enhancing separations (18). Some work has been reported using completely inorganic mobile phases for separations on reversed-phase columns with elution times varying from over 30 to <9 min (19–22). Ding *et al.* directly compared ion exchange, ion exclusion and RP chromatography for the separation of aliphatic organic acids. From this study, it was concluded that while retention times were longer than RP-LC, narrower peaks and improved resolution were observed with IELC. Ion exchange provided less interference than the other two methods (23).

The combination of anion exchange chromatography with mass spectrometry (MS) detection was shown to be very effective for the separation of 20 organic acids in ~35 min using a four-step gradient mobile phase program of NaOH from 5 to 20, 20 to 40, 40 to 60 and finally 60 mM. Various juice and wine samples were analyzed and MS permitted quantitative determination of some overlapping peaks (24).

Ion chromatography, with suppressed conductivity detection, is an important established method for the separation of either inorganic anions or organic acids or mixtures of both. The separation of an eight-component mixture of dicarboxylic acids such as glutaric, malic, tartaric, malonic, *trans*-β-hydromuconic, fumaric, oxalic and *trans*,*trans*-muconic acids was done in ~25 min using an aminated latex-based pellicular anion exchange column and an isocratic carbonate mobile phase (25). The presence of an organic solvent in the mobile phase caused swelling of the latex resin reducing available ion exchange and hydrophobic interactions. Using the same type of pellicular anion exchange column, separations of various five, eight or nine component combinations of these organic acids (formate, acetate, propionate, lactate, pyruvate, oxalate, malonate, succinate, tartrate, fumarate, and maleate) plus the inorganic anions Cl^-, NO_3^- and SO_4^{2-} were done in ~10–15 min using an isocratic NaOH mobile phase of either 5, 25 or 50 mM (26). The retention mechanism was primarily ion exchange; however, ion exclusion retention of the organic acids with the underlying sulfonated surface layer was also thought to play a role. This topic as well as the impressive capability of hydroxide gradient elution of complex organic acid and inorganic anion mixtures using similar columns has been reviewed (27).

IELC provides several advantages over RP-HPLC including the use of an aqueous mobile phase with little or no organic modifier for isocratic elution. Aqueous mobile phases are more environmentally friendly. Several detection methods can be used with IELC, including UV-VIS absorbance (28–30), conductivity (29–32), refractive index (28, 32) and MS (33) detection. IELC can be performed on a variety of columns. Most often separations are performed on strong (3, 13) and weak (34) ion exchange columns. Normal phase silica columns have also been used (2, 8). The most popular columns for organic acid separations are strong cation exchange columns, in which the polystyrene-divinylbenzene (PS-DVB) resin provides a high concentration of functional groups with high porosity (6). Wang *et al.* compared a strong cation exchange resin column to a C18 resin column and observed differences in peak resolution and retention order. In this study, it was concluded that the PS-DVB cation exchange resin provided higher resolution, sensitivity and efficiency and was therefore better suited for their separation than the RP column (35). Silica columns were also investigated for IELC separations of carboxylic acids (2, 8). Silica is recognized as a useful packing material for HPLC due to its physical and chemical stability, and the silanol group acts as a weak cation exchanger at a pH greater than 2, such that the separation mechanisms on silica columns are mainly based on ion exclusion (2). Previous work reports a reversed-phase column modified by nonionic surfactant, Triton X-100 followed by the cetylpyridinium ion, for the separation of organic and inorganic anions by ion exchange chromatography (36). Šlais was the first to use a surfactant-modified reversed-phase column for IELC separations. IELC separations of aromatic carboxylic acids were performed on a sodium dodecyl sulfate (SDS)-coated C18 column with an ammonium sulfate mobile phase (37). Mansour *et al.* also used a SDS-C18 column to isocratically separate a mixture of one aliphatic and two aromatic carboxylic acids under IELC conditions using an aqueous mobile phase (38). However, use of surfactant-modified reversed-phase columns for IELC of aliphatic carboxylic acids has not been studied in detail.

RP and IELC remain popular methods for the separation of aliphatic acids today. Ion exclusion columns with strong cation exchange stationary phases, such as Agilent Hi-Plex H and Phenomenex Rezex ROA, are recommended for organic acid separations. Separations on these columns tend to exhibit longer retention times than separations performed on aqua-C18 columns. Manufacturers recommend the use of aqua-C18 or ion exchange columns for such separations. Aqua-C18 columns are RP columns with polar endcapping to provide higher stability of the stationary phase in 100% aqueous mobile phases. Columns, such as Thermo Aquasil C18, Hitachi LaChrom C18-AQ and Phenomenex Synergi Hydro-RP, are commercialized as RP columns with aqua endcapping for more efficient organic acid separations. Endcapping of the stationary phase can greatly influence the separation such analytes. Less effective separations can be expected on columns with more hydrophobic endcapped stationary phase. To our knowledge, aqua-C18 columns are not commercially available for ultra-high performance liquid chromatography (UHPLC) separations. Phenyl-RP and pentafluorophenyl (PFP)-RP UHPLC columns are available but are not specifically designed for organic acid separations; such columns are also very expensive. A few UHPLC ion exchange columns for proteins have just recently become commercially available but are very expensive (39). These packings are based on a nonporous PSDVB core particle that is encapsulated by a hydrophilic polymer layer to eliminate nonspecific binding. Both weak and strong cation exchange groups have been bonded to this hydrophilic layer. However, to the extent of our knowledge, there are currently no IELC columns designed for UHPLC.

In this study, we developed a SDS dynamically modified reversed-phase column that is capable of separating short chain aliphatic acids under standard HPLC and UHPLC conditions. The SDS-modified column was applied to the separation of short chain aliphatic acids in beverage samples. The use of surfactant-modified columns allows for potential flexibility and customization of the column for the needs of the separation, via variation of the modifying surfactant. The developed method allows for UHPLC separations of aliphatic carboxylic acids.

Instrumentation and reagents

Chromatographic separations were performed on a Dionex Ultimate 3000 UPLC system, equipped with an Ultimate 3000 RS diode array detector, interfaced with Chromeleon software. The separation was performed on a Phenomenex Kinetex XB-C18 HPLC column (150 mm × 4.6 mm, 2.6 µm silica, 10 nm pore size) held at 20°C by the column compartment oven. This stationary phase (10% carbon load) has di-isobutyl side chains that can effectively shield the silica surface (200 m^2/g) providing an acidic pH stability of 1.5. A 20-µL injection volume was used for all experiments. UV detection of the analytes was performed at 210 nm. Absorbance measurements were performed on a Hewlett-Packard Model 8453 UV-Vis photodiode array spectrophotometer.

Sulfuric acid, purchased from Fisher Scientific (Pittsburgh, PA, USA), was diluted and used as the eluent in all separations, unless otherwise stated. SDS was obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate and citric acid were purchased from Fisher Scientific (Pittsburgh, PA, USA). Fumaric acid potassium salt, L-lactic acid, maleic acid disodium salt, and L-malic acid disodium salt were procured from Sigma-Aldrich (St. Louis, MO, USA). Other aliphatic carboxylic acids were obtained from a variety of sources. Analyte solutions were stored at 4°C when not in use.

Methods

Sample preparation

All solutions in this work were made with distilled and deionized water, passed through a Milli-Q (Millipore, Bedford, MA, USA) water purification system. Three short chain aliphatic acid mixtures were made for method development. The three mixture compositions are listed in Table I, with their acid dissociation constants (pK_a values). Chemical structures for all aliphatic acids studied can be found in Figure 1.

Seven beverages were analyzed for organic acid content using the developed IELC method. Five of the beverage samples were soft drinks, with controlled matrices, making for easier separation and analysis. Two of the beverage samples were fruit juices, with more complex matrices and aliphatic carboxylic acid analytes. All beverage samples were diluted 1:20 (v/v) with water and filtered through a 0.22-µm syringe filer. Carbonated beverage samples were heated on low heat to remove carbonation before dilution and filtration. Beverage contents, including organic acids, colored dyes and sweeteners, are listed in Table II.

Table I. Organic Acid Mixture Compositions with Corresponding Acid Dissociation Constants

Organic acid mixture	Carboxylic acid	pK_{a1}	pK_{a2}	pK_{a3}
Mix 1	Oxalic	1.23	4.19	6.40
	Citric	3.13	4.76	
	Malic	3.40	5.11	
	Succinic	4.16	5.61	
Mix 2	Malonic	2.83	5.69	
	Tartaric	2.98	4.34	
	Lactic	3.86		
	Acetic	4.75		
Mix 3 (isomer mix)	Maleic	1.83	6.07	
	Fumaric	3.02	4.39	

Column modification

For IELC separations, the C18 column was dynamically modified with SDS, by running a 10 mM solution of SDS through the column for 3 h at 0.3 mL·min^{-1} and then rinsing the column thoroughly with water for 2 h at 0.6 mL·min^{-1}.

SDS quantification

SDS was stripped off the column using HPLC grade methanol at a flow rate of 1 mL min^{-1} for 3 h. The methanol solution was collected in a round-bottom flask and evaporated via rotary evaporation at 30°C, leaving the SDS collected off the column. This solid SDS was re-dissolved in water. The surfactant quantification procedure used ion-pair formation of SDS by methylene blue and subsequent extraction by chloroform for spectrophotometric measurement (40). The SDS standards were prepared from a 10-ppm stock solution. One milliliter sample volumes of the standards were added to conical centrifuge tubes with 0.5 mL of 0.05% methylene blue stock solution in 0.7 mM sodium phosphate buffer, pH 7.20. A 3 mL volume of HPLC grade chloroform was added to each tube and inverted four to six times. Tubes were touched to a Vortex mixer to ensure adequate mixing. The aqueous and organic phases were separated by centrifugation for 3 min at 2,500 rpm at ambient temperature. Samples were allowed to stand for ~10 min before absorbance measurements of the chloroform layer were taken. Absorbance was monitored at 295 and 655 nm. This procedure was repeated for three individual column coatings.

Results

Reversed-phase HPLC

Prior to dynamic modification with SDS, the short chain aliphatic carboxylic acids mixtures, given in Table I, were analyzed on the unmodified C18 column for characterization of analyte retention

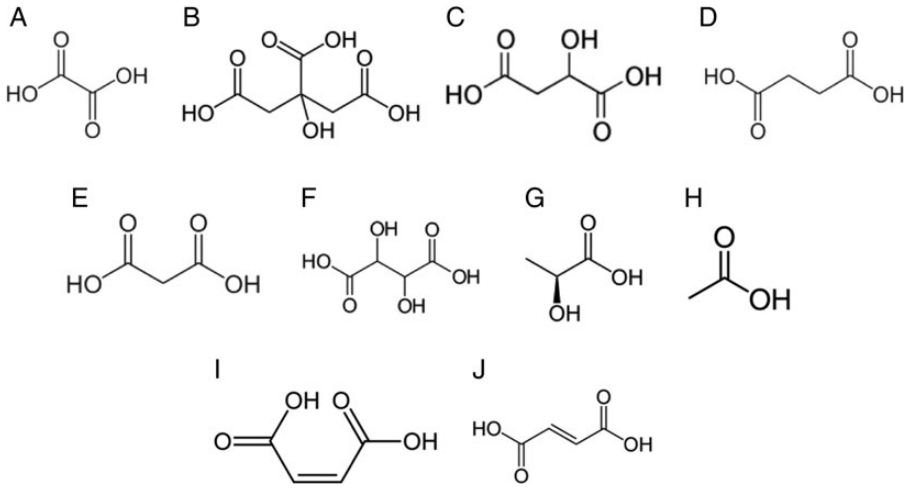

Figure 1. Structure of aliphatic carboxylic acids including, oxalic (A), citric (B), malic (C), succinic (D), malonic (E), tartaric (F), lactic (G), acetic (H), maleic (I) and fumaric (J) acids.

Table II. Contents of Beverages Analyzed by the Developed IELC Method

Beverage	Organic acids	Sweetener	Caffeine	Dyes
White grape juice	Quinate Lactate Acetate Galactuonate Succinate Malate Tartrate Maleate Fumarate Oxalate Citrate	Natural sugars	No	No
Apple juice	Quinate Lactate Acetate Galactuonate Succinate Malate Tartrate Oxalate Citrate	Natural sugars	No	No
Sprite®	Citrate Benzoate	High-fructose corn syrup	No	No
Sprite Zero™	Citrate Benzoate	Aspartame Acesulfame potassium	No	No
Fanta Strawberry®	Citrate Benzoate	Sugar	No	Red 40
Fanta®	Citrate Benzoate		No	Red 40 Yellow 6
Diet Sunkist®	Citrate Malate Benzoate	Aspartame Acesulfame potassium	Yes	Red 40 Yellow 6

behaviors in the RP mode. Mixtures were separated with a H_2O mobile phase adjusted to pH 3.0 with dilute sulfuric acid at a flow rate of 0.6 mL min^{-1}. Representative chromatograms are shown in Supplementary data, Figures S1–S3. Multiple peak forms for certain individual organic acids, particularly fumaric and maleic, make the chromatograms difficult to interpret. The range of retention factors was quite low: 0.1–0.8 for mixture 1, 0.1–0.4 for mixture 2, and 0.4–2.3 for mixture 3. The retention order was predicted based on the hydrophobic mechanism. In organic acid mixture 1, oxalic acid eluted first being the smallest and likely only charged molecule of the mixture. Malic acid followed in retention order, having no charge and being slightly more hydrophobic than oxalic acid. Citric acid preceded malic acid. The secondary citric acid peak results from two species of citric acid existing at pH 3.0. Finally, succinic acid was eluted last in this mixture, because it is the most hydrophobic and has the highest pK_a. The selectivity factors for peak pairs citric/malic acid and succinic/citric acid were estimated to be 2.5 and 1.8, respectively. Succinic acid is well resolved and shows a symmetric peak shape, permitting an effective plate count (N_{eff}) of ~1,040 to be calculated. Acetic acid, of organic acid mixture 2, eluted first due to the small size and its hydrophilic nature. It can be surmised that the charge and hydroxyl groups account for the elution of tartaric acid before lactic acid, which is a much shorter but uncharged acid. Although malonic acid would be charged at pH 3.0, it is the most hydrophobic acid of the mixture and therefore is eluted last in the RP mode. Fumaric and maleic acids are structurally similar, being geometric isomers, however, fumaric acid elutes before maleic acid in the RP mode. Both acids were found to have minor secondary species at pH 3.0. However, RP separation of the aliphatic acids was generally found to be unsatisfactory with weak retention and nonbaseline resolution for mixtures 1 and 2, while mixture 3 showed multiple broad and asymmetrical peaks. The hydrophobic endcapping on the Kinetex column is thought to contribute to poor separation and weak retention of these organic acids compared with previous reports of RP separations. RP-HPLC proved to be an inefficient separation method for short chain carboxylic acids and therefore characterization of the SDS modified C18 column for IELC follows.

Modified column characterization

Sample capacity of SDS-coated column

The sample capacity of the SDS-C18 column was examined to prevent overloading of the column and allow for efficient separations. A test solution mixture containing acetic, lactic and malonic acids, with concentrations ranging from 0.01 to 100 mM, was used for capacity determination. Chromatograms of these test mixtures are shown in Supplementary data, Figure S4. Separation of the carboxylic acids was achieved in <5 min and adequate resolution remained constant. Retention factor and N_{eff} values for the three organic acids were used as indicators of IELC sample capacity (Figure 2), a parameter rarely studied in the literature. In general, a significant drop in both retention factor and plate count occurred at a log mM value of 1.0 for all three test compounds. Sample capacity of the SDS modified column was therefore determined to be near 10 mM for a 20 µL injection volume or 0.2 µmol.

van Deemter curve

A van Deemter curve was generated for the SDS-C18 column, using tartaric acid and organic acid mixture 2 (Figure 3). If the A and C_s

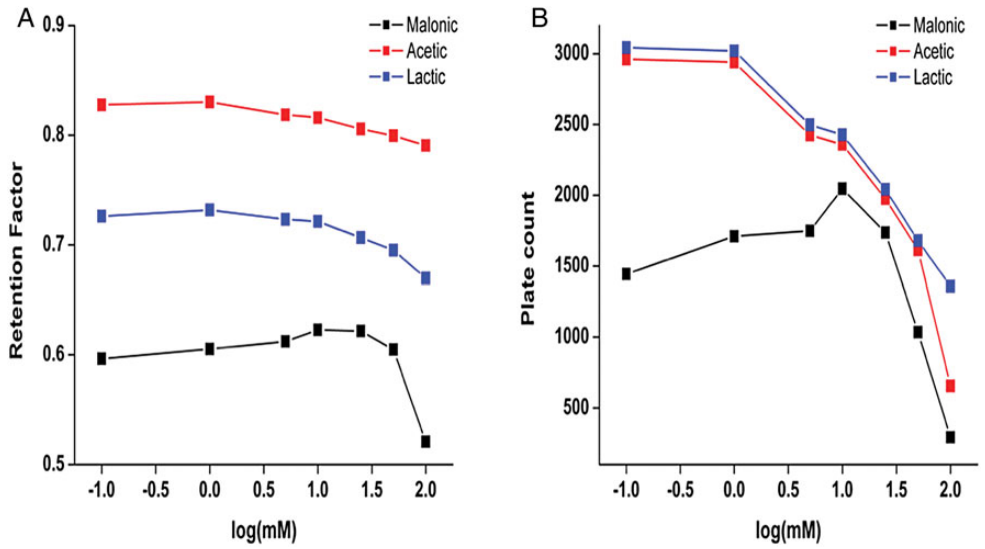

Figure 2. Column sample capacity of the SDS coated column as measured by retention factor (A) and effective plate count N_{eff} (B), for three organic acids. For (A): malonate (bottom), lactate (middle) and acetate (top). For (B): malonate (bottom), acetate (middle) and lactate (top). Log(mM) = log[analyte acid]. Points connected for clarity. This figure is available in black and white in print and in color at *JCS* online.

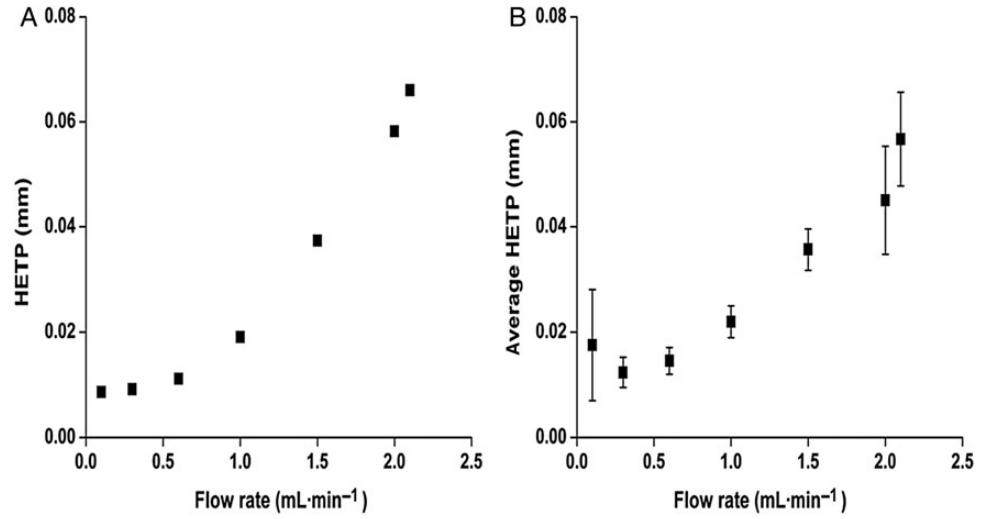

Figure 3. van Deemter curves for SDS modified column for the plate height of tartaric acid (A) and the average plate height for all acids in organic mix 2 (B). H values given are averages of each of the four analytes in mixture 2, with relative standard deviations between 10.9 and 60.1%.

van Deemter terms are neglected, the ideal plate height can be predicted to be about two to three times the particle diameter (d_p). The plate height of 0.012 mm found for the optimum flow rate of 0.3 mL min^{-1} was somewhat higher than the ideal. When considering analysis time, a practical flow rate was determined to be 0.6 mL min^{-1} for a plate height of 0.015 mm.

Pressure stability of SDS-modified column

The backpressure of the SDS-C18 column was tested as a function of the flow rate to ensure pressure stability when obtaining chromatograms under UHPLC conditions. The relationship between flow rate (x) and backpressure (y) demonstrated two linear trends (Supplementary Figure S5), described in detail in the Supplementary data. The Kinetex column was reported to have pressure limit of 8,000 psi. Under UHPLC separations, a column backpressure of 7,500 psi corresponded to a flow rate of 2.1 mL min^{-1}.

Quantification of SDS on the modified column

The SDS determination is based on ion-pair formation between SDS and methylene blue in a 1:1 ratio. This complex is extracted from the aqueous phase into the organic solvent, while free methylene blue remains in the aqueous phase. The colorimetric determination of the dye in chloroform can therefore be used to quantify SDS (40). The calibration curve used for the SDS-methylene blue extraction can be found in Supplementary data, Figure S6. An average of 99.91 ± 12.48 mg (n = 3) of SDS was determined using the 655 nm absorbance for the three individual column modifications. Similarly, an average of 96.00 ± 15.83 mg (n = 3) was determined using the 295 nm absorbance. Previous methods cite only the 655 nm wavelength; however, quantification results using both wavelengths were in fairly good agreement and should be considered. Therefore the average value of 98 mg was used to determine there was 0.39 mmol SDS coated on the stationary phase of this 150 × 4.6 mm ID column packed with

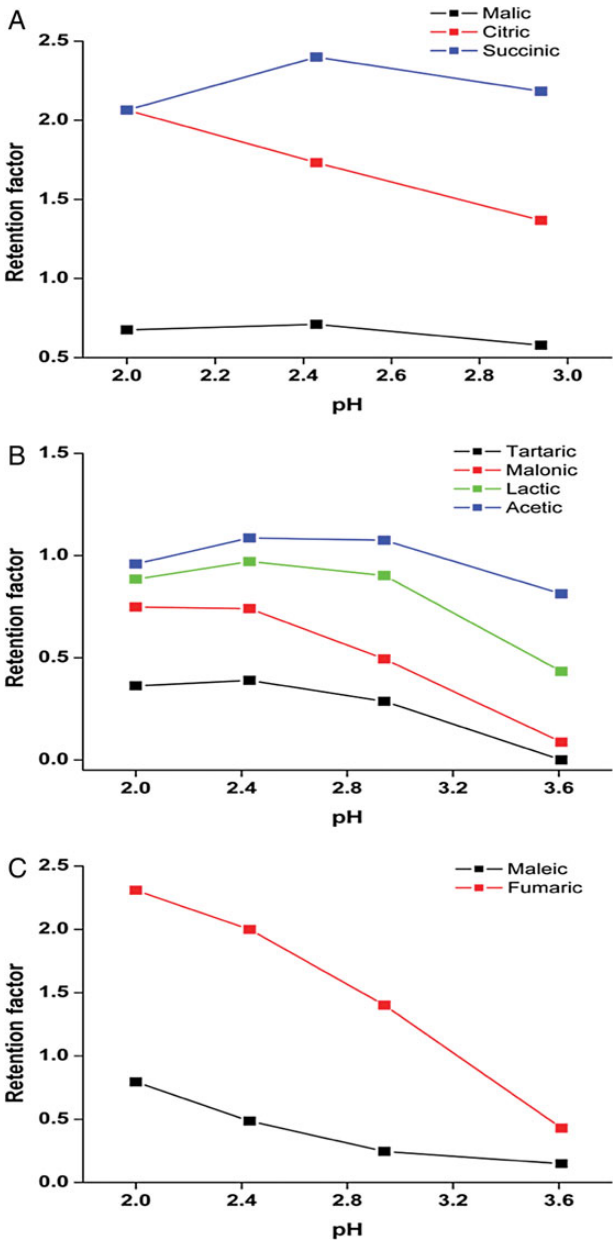

Figure 4. Effect of pH on retention factor, organic acid mixtures 1, 2 and 3 in (A), (B) and (C), respectively. Organic acid mixture 1: malic (bottom), citric (middle) and succinic (top). RSD values generally between 0.17 and 8.8%. Organic acid mixture 2: tartaric (bottom), malonic (middle), lactic (high) and acetic (top). RSD values generally between 0.02 and 1.4%. Organic acid mixture 3: maleate (bottom) and fumarate (top). RSD values generally between 0.57 and 9.7%. Points connected for clarity. This figure is available in black and white in print and in color at *JCS* online.

2.6 mm C18 silica particles. This value is similar to the coverage of 0.25 mmol SDS in a 150 × 4.6 mm ID column packed with 5 mm C18 silica particles, which was reported by Ito and co-workers (41).

Optimization of IELC parameters

Because the pH of the solution determines the degree of ionization of the analytes, it was very important to examine the effect of pH on the separation mechanism. The change in retention factor was studied over the pH range of 2.0–3.6 using 1.84 mM H_2SO_4 adjusted with either dilute NaOH or dilute H_2SO_4 (Figure 4). It was found that retention decreases significantly toward the higher end of the pH range evaluated. Due to the proximity of the pH to the organic acid pK_a, the analytes will be more ionized and repelled by the Donnan membrane. However, this effect is variable depending on the analyte ionization constant and hydrophobicity. A description of the IELC retention factors taken at pH 2.94, provided to permit a comparison to the previous RP-HPLC separation of organic acids at pH 3.0, follows. Retention factor ranges were consistently higher for the more polar acids: 0.2–1.4 for mixture 1 and 0.3–1.1 for mixture 2. For the unsaturated nonpolar acids (mixture 3), the retention factor range of 0.2–1.4 was less than RP-HPLC, but peak shape was substantially better. The selectivity factors α for peak pairs citric/malic acid and succinic/citric acid were 2.4 and 1.6, respectively, comparable to those for RP-HPLC. The N_{eff} value of ~4,440 for succinic acid was significantly better by a factor of four than that for RP-HPLC. However, based on the plot in Figure 4, a pH of 2.43 was determined to be optimal for IELC of these organic acid mixtures.

The effect of sulfuric acid mobile phase concentration on the retention mechanism was examined without pH adjustment, leading to the study of a combined effect of ionization and ionic strength on the retention mechanism. Sulfuric acid concentration was varied from 0.184 to 1.84 mM with corresponding pH values of 3.43 to 2.43, respectively, for all organic acid mixtures (Figure 5). This pH range is not too far from the pK_{a2} value of 1.92 for sulfuric acid. In general, most organic acid retention factors were not influenced by changes in sulfuric acid concentrations, but more so by the changes in pH, showing similar trends to the study of pH on retention factor.

The effect of sulfuric acid concentration was studied to determine the effect of ionic strength on the strength on the separation mechanism. This effect was studied by retention factor with organic acid mixture 3 (isomer mixture), seen in the Supplementary data, Figure S7. The concentration was varied from 0.184 to 18.4 mM at a constant pH of 2.00. As expected, no appreciable change in retention factor was observed as the ionic strength was varied.

Organic acid mixture 3 was used to study the effect of column temperature on the separation. While retention times were slightly enhanced at 30°C, no significant changes were observed in peak shape and resolution when compared with 20°C. Integrity and stability of the SDS-coated column could be of concern at elevated temperatures; therefore, 20°C was chosen as the column temperature for the remainder of the experiments.

IELC chromatograms

Three test mixtures of aliphatic organic acids, listed in Table I, were used to study the SDS-C18 column for ion exclusion separation under HPLC conditions. The mobile phase consisted of 1.84 mM H_2SO_4 (pH 2.43) at a flow rate of 0.6 mL min^{-1}, as determined from optimization experiments. These mixed acid solutions showed good separation with high resolution and no broad peaks under IELC conditions as seen in chromatograms A, B and C of Figure 6 (left column).

The same SDS-C18 column was used to separate the organic acid mixtures under UPLC conditions. A mobile phase of 1.84 mM H_2SO_4 was used at flow rate of 2.1 mL min^{-1}, as determined from optimization experiments. The chromatograms (right column of Figure 6A–C) demonstrated good separation in approximately one-third the time of separation by HPLC; however, resolution suffered some under these conditions. Figure 6 does show a shoulder peak for citric acid in both standard IELC and IELC in the UHPLC mode; possibly these

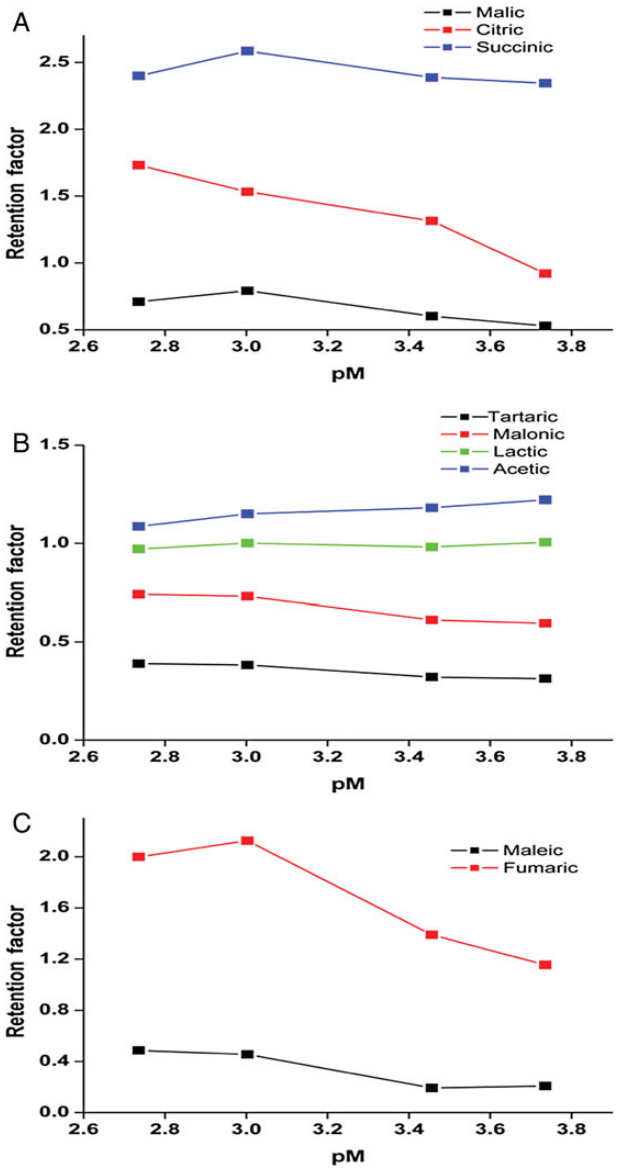

Figure 5. Effect of H_2SO_4 eluent concentration on retention factor of organic acid mixtures 1 (A), 2 (B) and 3 (C). pH was not controlled in this experiment. pM = $-\log[H_2SO_4]$. Organic acid mixture 1: malic (bottom), citric (middle) and succinic (top). Organic acid mixture 2: tartaric (bottom), malonic (middle), lactic (high) and acetic (top). Organic acid mixture 3: maleate (bottom) and fumarate (top). Points connected for clarity. This figure is available in black and white in print and in color at *JCS* online.

are different forms of this tricarboxylic acid. The IELC in the UHPLC mode chromatogram does show an extra peak that may be derived from malic acid; we are unsure of the origin of this peak and why it is not evident in the standard IELC chromatogram. Although hydroxide gradient ion exchange separations of organic acids have been shown to be effective (24), some peak pairs that we have resolved easily are difficult to distinguish by ion exchange. Malic and succinic acid as well as malonic and tartaric acid peak pairs were unseparated or significantly overlapped (24). Dicarboxylic aliphatic acid peak pairs of malic/succinic acid and maleic/fumaric acid were also not resolved on a polystyrene–divinylbenzene anion exchange column (25).

Reproducibility of SDS coating

Previously, we indicated that a reproducible amount of SDS could be adsorbed by the column as ascertained by the methylene blue colorimetric method. However, evidence of chromatographic reproducibility was also important to know and such reproducibility of the SDS-C18 column was tested twice throughout the course of these experiments. The column was stripped using methanol and re-modified with SDS in the same manner described in the "Methods" section. After re-coating the column, aliphatic carboxylic acid mixture 3 was used to test the reproducibility of the modified column by checking for retention factor and peak symmetry changes. This mixture was chosen, because it is the simplest mixture, with only two analytes, and great peak resolution. As previously discussed, these analytes demonstrate a unique property in changing retention order depending on the dominant retention mechanism. This switch in retention order based on the separation mechanism (IELC or reversed-phase) proved to be a useful indicator of the SDS condition within the column. An observed change in retention order suggested that the SDS coating was stripped away exposing C18 and allowing reversed-phase retention to dominate. This signaled that the column required re-modification. Figure 7 demonstrates this concept by displaying a chromatogram of organic acid mixture 3 while the SDS coating is intact (Figure 7A), separation by IELC, and a comparison chromatogram of the same mixture when the SDS coating is damaged (Figure 7B), leading to separation by reversed-phase chromatography. The SDS coating of a single column was also found to be very reproducible, as demonstrated by the organic acid mixture 3 chromatograms in Figure 6C (left column) and Figure 7A. The chromatogram in Figure 7A was collected on the same column ~2 months after the chromatogram in Figure 6C. Both chromatograms show excellent separation and peak shapes and similar retention times.

Application to beverage samples

Seven beverages were qualitatively analyzed for aliphatic carboxylic acid content using the developed IELC method. The samples and their contents, including organic acid content, have been tabulated in Table II. Using the SDS-C18 column with the optimized method, components within the samples were separated and identified, including not only aliphatic carboxylic acids but also sweeteners and caffeine. In the white grape juice sample, six organic acids (oxalic, galacturonic, tartaric, malic, acetic and citric) were separated and identified as major components (Figure 8A). The other peaks (2, 7 and 10) were small and poorly resolved; identification is only tentative. Similarly, three organic acids (galacturonic, malic and acetic) were separated and identified as major components in the apple juice sample (Figure 8B). The other peaks (1, 2, 4, 5, and 7) were small and poorly resolved; identification is only tentative.

Soft drink samples contained varying components that have potential to interfere with analysis of the carboxylic acids. Therefore, we tested drinks with dyes, caffeine and different types of sweeteners to examine their effect on the separation and identification of short chain carboxylic acids. The chromatograms of the soft drink samples are given in Figure 9. The simplest of these soft drinks was Sprite®, which does not contain caffeine, nor artificial dye, and is sweetened using high-fructose corn syrup. Three clear peaks are present, the first, at 1.94 min, was assigned as an unidentified component of the sample, likely due to the natural flavorings like lemon oil. The second and third peaks correspond to glucose/fructose (2.55 min) and citric acid (6.24 min), respectively. These results agree with the ingredients listed for the beverage. Fanta® and Fanta® Strawberry contain

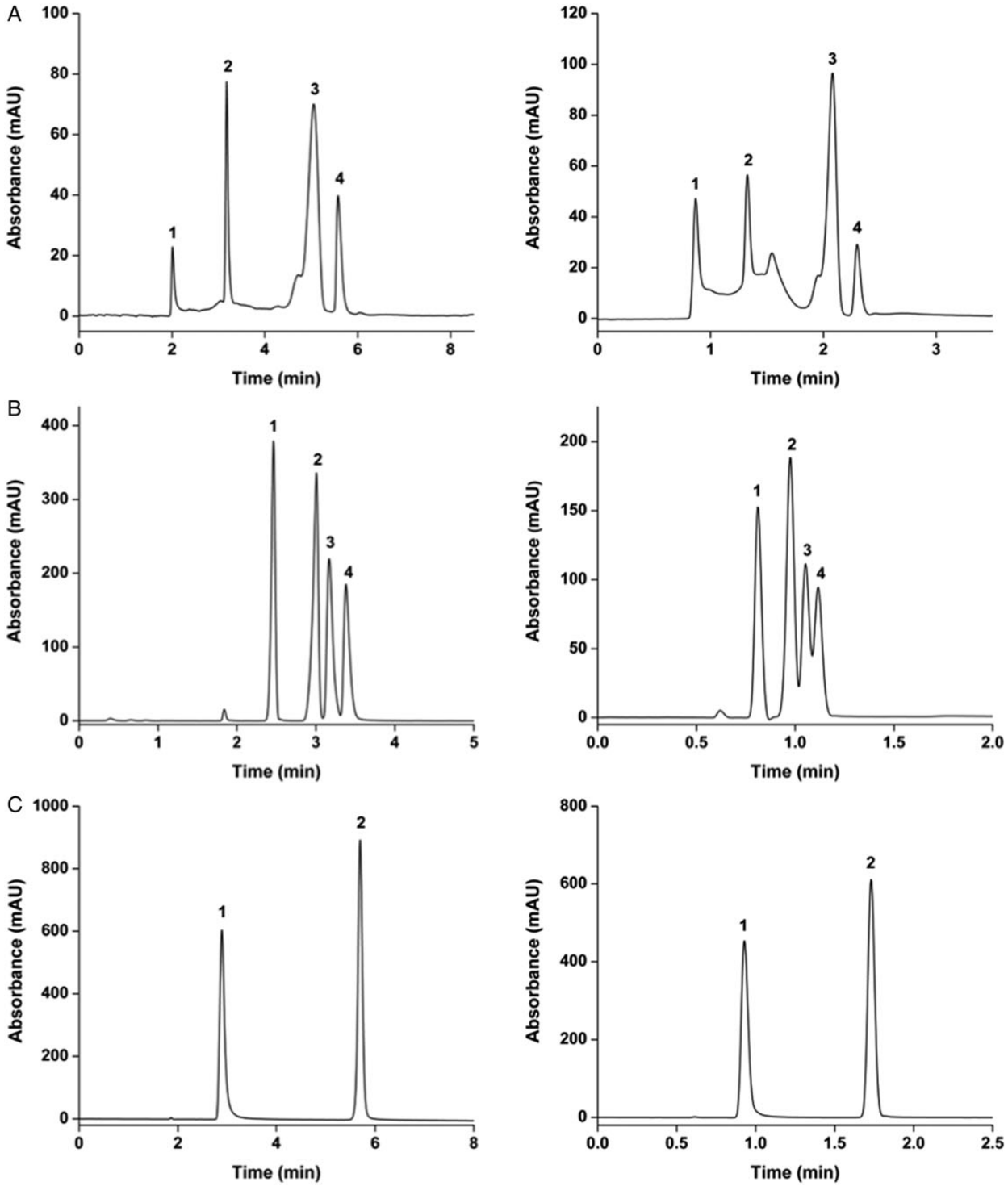

Figure 6. Chromatograms of organic acid mixtures 1 (A), 2 (B) and 3 (C), all components 1 mM, on the SDS-C18 column, with a mobile phase of 1.84 mM H_2SO_4 (pH 2.43), at flow rates 0.6 mL·min^{-1} (left column) and 2.1 mL·min^{-1} (right column), corresponding to approximate back pressures of 2,600 and 7,500 psi, respectively. Peak assignments for organic acid mixture 1: (1) oxalic acid, (2) malic acid, (3) citric acid and (4) succinic acid. Peak assignments for organic acid mixture 2: (1) tartaric acid, (2) malonic acid, (3) lactic acid and (4) acetic acid. Peak assignments for organic acid mixture 3: (1) maleic acid and (2) fumaric acid.

artificial red and yellow dyes, which Sprite® did not. These chromatograms in Figure 9 show three main peaks with the same retention times as those in Sprite® and can be attributed to the same carboxylic acids. The dyes were found not to interfere with sample analysis. Diet Sunkist® not only contains artificial dyes, but also caffeine and aspartame. This chromatogram in Figure 9 displays four peaks. The first peak, at 1.84 min, was assigned to an unidentified component. Malic acid is listed as an ingredient in Diet Sunkist®; it is not an

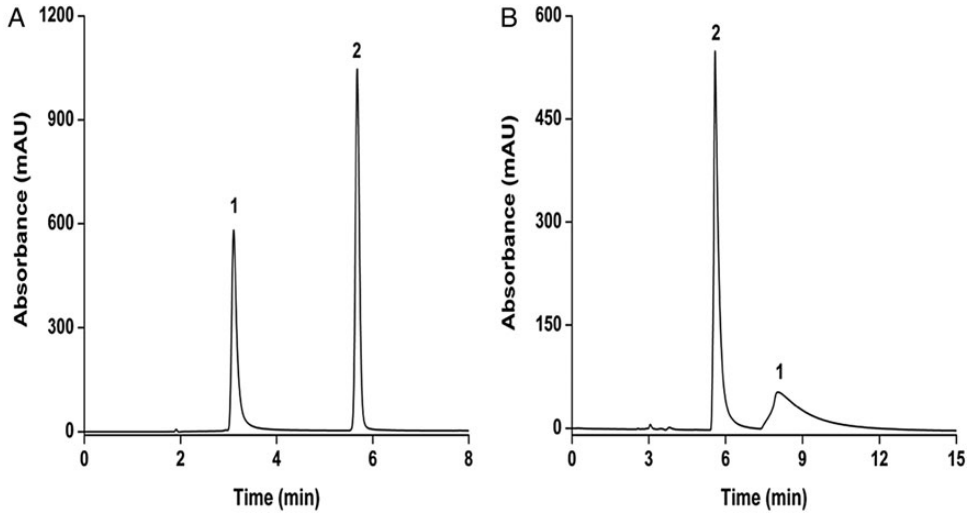

Figure 7. Chromatograms of organic acid mixture 3, with a mobile phase of 1.84 mM H_2SO_4 (pH 2.43), at flow rates 0.6 mL min^{-1}, under good SDS coating (A) and degraded SDS coating (B). Maleic acid, peak 1, is eluted first under good SDS coating conditions, while fumaric acid, peak 2, is eluted second. When degradation of SDS coating on the column occurs fumaric acid is eluted first, peak 1, followed by maleic acid, peak 2.

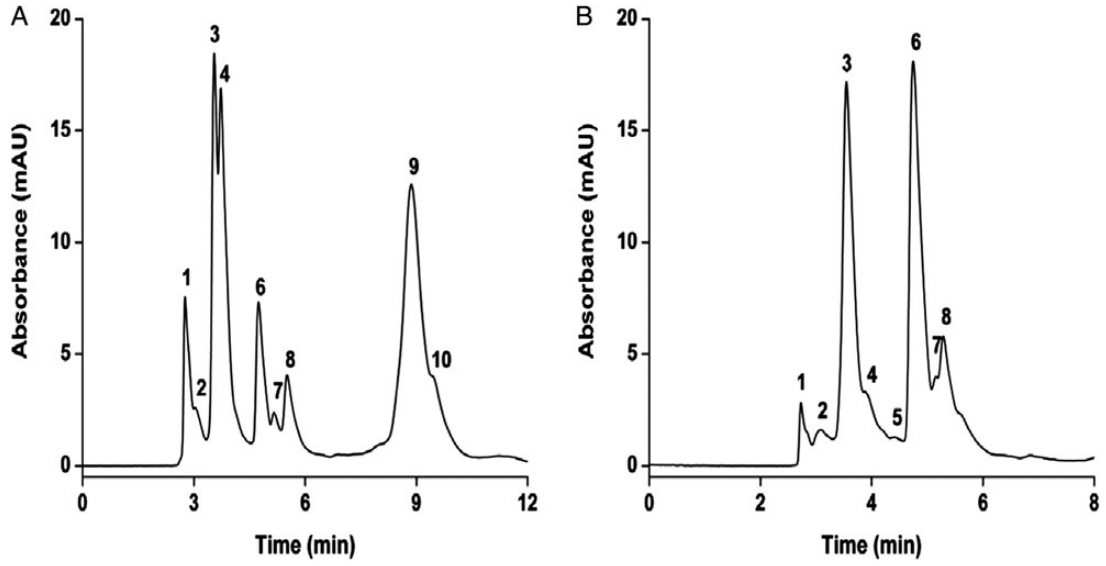

Figure 8. Chromatograms of fruit juice samples, white grape juice (A) and apple juice (B), on the SDS column, with a mobile phase of 1.84 mM H_2SO_4 (pH 2.43), at a flow rate 0.4 mL min^{-1} . Positive peak assignments for white grape juice (A): (1) oxalic acid, (3) galacturonic acid, (4) tartaric acid, (6) malic acid, (8) acetic acid, (9) citric acid. Tentative peak assignments: (2) sugar (fructose or glucose), (7) lactic acid, (10) fumaric and/or succinic acid. Positive peak assignments for apple juice (B): (3) galacturonic acid, (6) malic acid, (8) acetic acid. Tentative peak assignments: (1) oxalic acid, (2) sugar (fructose or glucose), (4) tartaric acid, (5) quinic acid, (7) lactic acid.

ingredient in the previous soft drink samples and was identified as the second peak at 3.20 min. The third peak, at 4.12 min, can be assigned to aspartame, the artificial sweetener in the soft drink. The final peak, at 5.87 min, was assigned as overlapping citric acid and caffeine peaks. Diet Sunkist® does contain a small amount of caffeine and was the only caffeinated beverage tested. The final soft drink tested was Sprite Zero™; the chromatogram is displayed in Figure 10. This chromatogram is much like that of Sprite®, with the glucose/fructose peak replaced by an aspartame peak. To demonstrate the separation capabilities of the column, this sample was also separated under UHPLC conditions with an elevated flow rate. The UHPLC chromatogram (Figure 10B) shows the same three peaks seen in the HPLC separation with retention times about three times faster while maintaining good resolution.

Both Figures 9 and 10 showed a weakly retained unidentified peak number 1 and consideration of their cause of formation was made. We noted in a previous study (41) that system peaks were not evident for the separation of alkali metals by ion exchange using a coated surfactant column. However, when SDS was added to the mobile phase to make a dynamic ion exchange column, a broad highly retained system peak was evident after the separation of alkali metals. In a different study (42), a system peak ascribed to the presence of hydronium

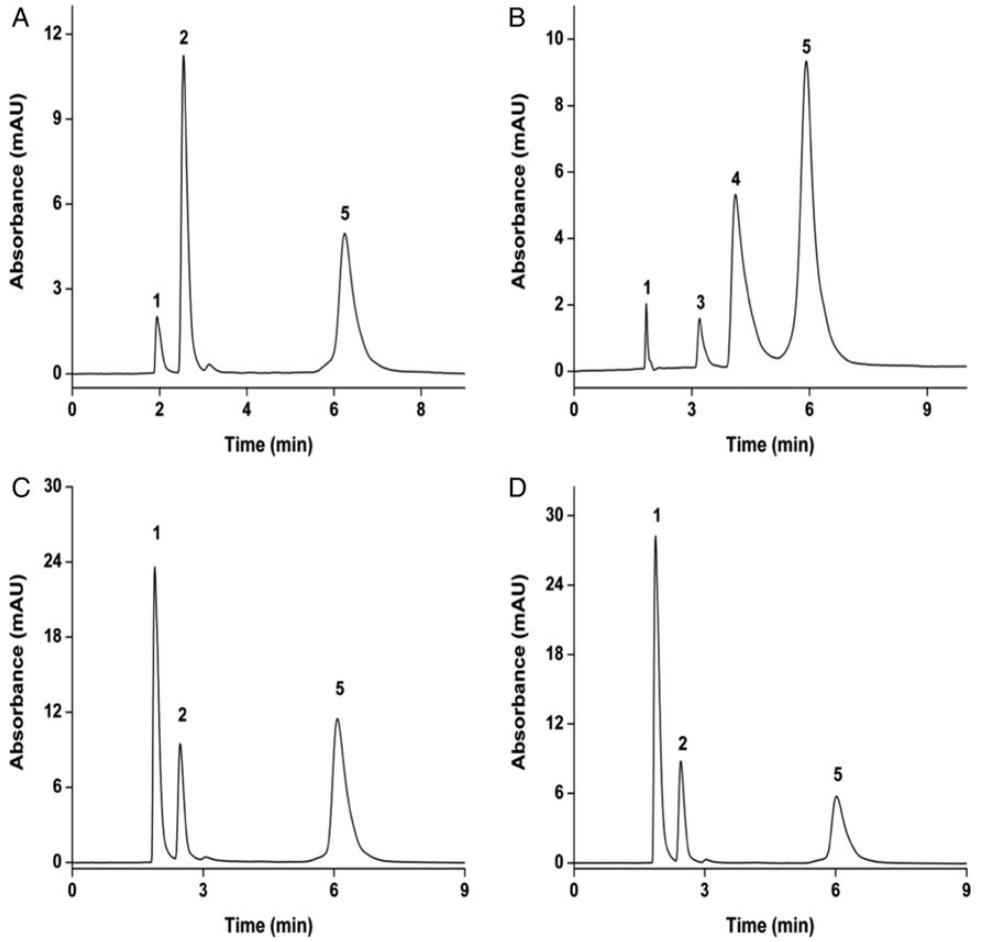

Figure 9. Chromatograms of soft drink samples, Sprite® (A), Diet Sunkist® (B), Fanta® (C) and Fanta® Strawberry (D), on the SDS-C18 column, with a mobile phase of 1.84 mM H_2SO_4 (pH 2.43), at a flow rate 0.6 mL min^{-1}. Peak assignments are as follows: (1) unidentified peak, (2) glucose or fructose, (3) malic acid, (4) aspartame and (5) citric acid (in Diet Sunkist caffeine overlaps).

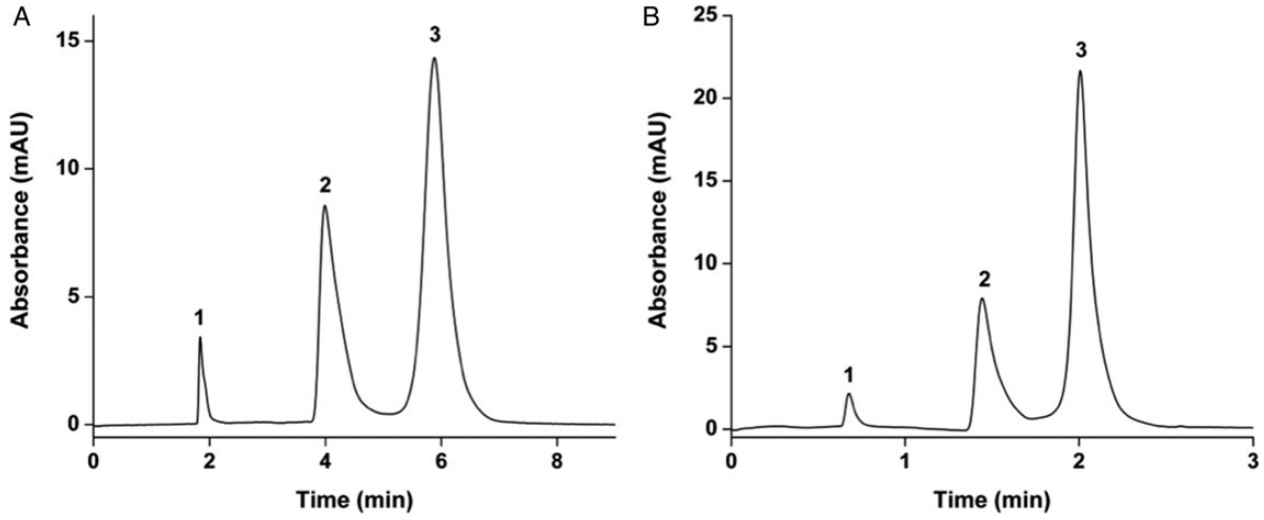

Figure 10. Chromatograms of Sprite Zero™ on the SDS-C18 column, with a mobile phase of 1.84 mM H_2SO_4 (pH 2.43), at flow rates (A) 0.6 mL min^{-1} and (B) 2.1 mL min^{-1}. Peak assignments are as follows: (1) unidentified peak, (2) aspartame and (3) citric acid.

ion, again using a dynamically coated SDS reversed-phase column for ion exchange of alkali metal ions. We believe that this peak may be due to a refractive index difference between the sample solvent and the mobile phase.

Discussion

Based on a more thorough review of the literature during the writing of this article, we have come to realize that the coating of a C18 stationary phase by SDS may depend on the concentration of the SDS solution being pumped through the column. We used a [SDS] of 10 mM, above the critical micelle concentration (CMC) of 8.3 mM. A spectroscopic study involving fluorescence of the 1,4-bis(*o*-methylstyryl)benzene probe and direct SDS infrared measurements has shown that using a [SDS] above the CMC appears to generate single monolayer coverage of SDS with good interpenetration of the SDS and C18 alkyl chains (43). Below the CMC, SDS is adsorbed with a different alkyl chain orientation resulting in a more dense organized monolayer, having about a factor of three more coverage than that initiated with [SDS] above the CMC. It seems an interesting future study would be to prepare IELC columns using different [SDS] to determine if the IELC retention process is changed for some organic acids. However, our SDS-coated C18 columns seemed to much more stable that those previously prepared using 1 mM [SDS] (41) and this is an important issue to be considered.

Retention order differences were noted between the RP and IELC mechanisms. The most apparent example of these differences is the switch in retention order of the organic acid isomers in mixture 3. In the RP mode, fumaric acid elutes before maleic acid, but in ion exclusion mode maleic acid elutes before fumaric acid. This reversed retention order can be explained by their pK_a values; maleic acid has a lower dissociation constant than fumaric acid. Because the acids are geometric isomers of each other, analyte hydrophobicity does not play as much of a role in the changes in retention order as pK_a does. At a pH of 2.43, maleic acid will be charged and will therefore be repelled by the Donnan membrane eluting faster than fumaric acid, which will interact with the occluded liquid and the underlying hydrophobic resin. A second example in mixture 2 of this shift in retention order is tartaric acid and malonic acid moving from the middle and end of retention order in reversed-phase mode, to the two least retained acids in the mixture in IELC mode. Both acids have a partial charge because the pH of the mobile phase is near both dissociation constants. Although tartaric acid has a higher pK_a than malonic acid, it is eluted first. This can be explained by the hydrophobicity of each acid; malonic acid is more hydrophobic than tartaric acid, which has some hydroxyl groups. This causes malonic acid to be retained slightly by hydrophobic interactions with the stationary phase. These changes in retention orders confirm the retention mechanism of IELC as being a mixture of hydrophobic adsorption and exclusion repulsion effects. It is apparent that hydrophobic interactions play a significant role in the retention mechanism.

We have compared our results to those found in a previous IELC study (44) of organic acids using a Dionex IonPac ICE-AS6 column (250 mm × 9 mm ID) which holds sulfonated crosslinked styrene–methacrylate–divinylbenzene 8 µm resin particles with an ion exchange capacity of 27 meq. Using a 1.6 mM pentafluorobutyric acid mobile phase at pH 2.8, 15 organic acids could be separated in ~35 min. The retention order of the same acids that we used in our study from low to high was oxalic, tartaric, citric, malic, lactic, acetic and succinic acids. Our retention order was similar with the exception of citric acid: oxalic, tartaric, malic, lactic, acetic, citric and succinic acid. A plot of retention factors for the corresponding acids determined on each column is shown in Supplementary data, Figure S8. The only major outlier is citric acid with stronger retention on the SDS-C18 column as compared with the Dionex resin column. Despite the differences in ion exchange capacity between the resin column and the SDS-C18 column, it seems an IELC retention model is operative in both studies.

This observation of change in retention order of maleic acid and fumaric acid in mixture 3 is a unique indicator as to the column condition and suggested the need for re-modification. Each time the reversed-phase column was re-modified with SDS, only slight changes in retention factor or peak asymmetry were observed. The mean retention factor and peak asymmetry for the fumaric acid peak were significantly different between the original and the re-coated SDS-C18 column ($P = 0.02$ and $P = 0.02$, respectively); however, this was not the case for maleic acid ($P = 0.19$ and $P = 0.43$, respectively). The original SDS coating of the column was stable for ~570 column volumes, while the second was stable for ~4,000; a column volume is about 1.1 mL. The justification for this discrepancy is that all UPLC separations were performed on the original SDS-coated column, while only HPLC separations were performed on the second coating. The addition of a low [SDS] in the mobile phase may improve the stability of SDS on the stationary phase without markedly affecting organic acid retention. We have some preliminary data indicating the [SDS] would have to be <0.05%. At this [SDS], k' values for most organic acids such as malonic, lactic and acetic are reduced by at least half, potentially jeopardizing peak resolution.

A polar nonionic surfactant was investigated for the UHPLC separation of organic acids in an attempt to create a stationary phase similar to that of an HPLC aqua-C18 column. The surfactant Brij-58, used in this study, has a polyoxyethylene structure with a C16 aliphatic tail. A UHPLC C18 column was modified with 10 mM Brij-58 using the same procedure described in the "Methods" section for SDS-C18 column modification. The aliphatic acid mixtures were separated on the modified column with limited resolution and broad peak shapes, as compared with our previous work using the SDS modified C18 column. Retention time shifts were noted in sequential chromatograms and are thought to be attributed to some instability of the Brij-58

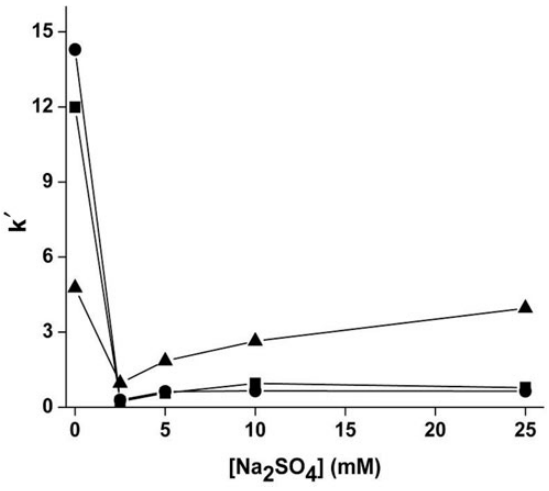

Figure 11. Mobile phase optimization on a SDS-C18 column for the separation of (triangle) 2-, (square) 3- and (circle) 4-hydroxybenzoic acid isomers. The effect of ionic strength was observed by retention factor (k') via addition of sodium sulfate to aqueous mobile phase. RSD values between 0.69 and 23%. Points connected for clarity.

coating, due possibly to the relatively polar polyoxyethylene group of the surfactant. It was determined that Brij-58 is not a viable alternative stationary phase modifier of a C18 column for the separation of aliphatic organic acids.

It is important to be aware of the potential challenges in separating aromatic acids or aromatic–aliphatic acid mixtures using this SDS-C18 IELC technique. Separation and analysis of hydroxybenzoic acid isomers are also important to the food and beverage industry. 2-, 3- and 4-Hydroxybenzoic acids were tested on the surfactant-modified reversed-phase column using chromatographic conditions determined in previous studies. Long retention times, ~120 min, and broad peak shapes were observed. It is hypothesized that the long retention times are caused by strong hydrophobic adsorption of the aromatic analytes to the stationary phase, with less electrostatic repulsive forces. The ionic strength and use of a mobile phase modifier was considered to improve the separation of these acids. The addition of small amounts of sodium sulfate to the mobile phase dramatically lowered retention factors, as shown in Figure 11. The 3- and 4-hydroxybenzoic acid isomers remained a challenge to separate, and peak shapes were not considerably improved. However, potential interference of aliphatic acid peak response by aromatic acids can be mitigated using sodium sulfate in the mobile phase.

Conclusion

We have shown that the separation of aliphatic carboxylic acids can be achieved by IELC using a SDS-C18 column in either the standard HPLC or UHPLC mode. The use of a surfactant-modified column allows for flexibility and minimizes the limitation of commercially available ion exchange columns for use in UHPLC. Sample capacity was determined to be 0.2 µmol. Separation conditions, like mobile phase flow rate, pH and concentration, were optimized. Under optimized HPLC conditions, a separation of four aliphatic carboxylic acids (oxalic, malic, citric, succinic) could be achieved in <6 min and in <3 min under UHPLC conditions. The separation of malonic, tartaric, lactic and acetic acids could be achieved in <4 min and in <2 min under UHPLC conditions. The separation of the isomeric carboxylic acids (maleic and fumaric) could be achieved in <6 min and in <2 min under UHPLC conditions.

The SDS-C18 column was used to separate major aliphatic carboxylic acid components in seven beverage samples, particularly in the simpler synthetic soda samples. At the present time, this IELC column is not well suited for profiling both major and minor organic acids in complex natural samples like fruit juice. Sports drink samples that often contain citric acid and may contain lactic acid could likely be analyzed using this method. Sports drinks may also contain calcium and magnesium ions, which could interact with the adsorbed SDS but probably only weakly.

IELC of aromatic acids has been recently reviewed (45) and use of a surfactant dynamically modified reversed-phase column for such mixtures has not been explored. Future work is expected to focus on how surfactant chain length and structure affect the retention mechanism, particularly for IELC of aromatic carboxylic acids that are positional isomers.

Supplementary data

Supplementary data are available at *Journal of Chromatographic Science* online.

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