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Liquid chromatography of short chain carboxylic acids using a glutamic acid surfactant coated C18 stationary phase

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ABSTRACT

A C18 column was modified with the anionic amino acid surfactant lauroyl-L-glutamate (LLG) to facilitate the separation of ten short-chain aliphatic carboxylic acids (oxalic, tartaric, malic, malonic, lactic, acetic, maleic, citric, fumaric, and succinic). The developed method was proven to be fast, versatile, and environmentally friendly. After the coating of the column using 1% LLG solution and optimizing chromatographic conditions such as pH and temperature, near baseline resolution of the ten carboxylic acids within 4 min with excellent peak shape at pH = 1.8 using 100% H_2O acidified with sulfuric acid was possible. Although the design of this stationary phase, with the hydrophilic group at the end of the alkyl chain, seems to be in contrast to such columns designed for a totally aqueous mobile phase that have a polar (often amide) group embedded near the silica surface, no evidence of phase collapse was noted. Linear relationships of ln retention factor (k) versus 1/Temperature (T) (van't Hoff plots) were generated for all the acids indicating a single retention mechanism was likely. As the pH of the mobile phase decreased, the analyte retention factors increased due to the increase of the fraction of the analyte with neutral charge (alpha zero). The surfactant amide linkage, being electron donating, increased the pKa of the more acidic carboxyl group of glutamic acid so both carboxyl groups were protonated (neutral) at pH 1.8. The exact nature of the retention mechanism is uncertain but there certainly seems to be a pronounced hydrophobic component due to the large difference in retention of fumaric acid and methyl fumarate at pH 1.8. In addition, eleven beverage samples were analyzed for their aliphatic carboxylic acid contents. The results showed that malic, fumaric, and citric acids were the most common carboxylic acids in natural beverages with concentrations as high as 6432 ppm of malic acid in organic apple juice, 64 ppm of fumaric acid in organic concord juice, and 6543 ppm citric acid in strawberry lemonade juice.

1. Introduction

Classically prepared reversed phase HPLC column packings based on octadecyl (C18) silane chemistry with silica microspheres and then endcapping with a short alkyl chain have excellent separation capability for moderately polar aromatic organic compounds such as pharmaceuticals using a mixed organic-aqueous mobile phase. However, the separation of highly polar organic compounds such as aliphatic organic acids often requires a 95–100% aqueous mobile phase. C18 phase collapse in highly aqueous environments can be a real issue causing short retention and poor separation of quite polar organic compounds [1]. To alleviate this problem, reversed phase stationary phases with a polar functional group such as an amide at a position on the alkyl chain near the silica surface have been developed and are known as polar embedded stationary phases [2]. This polar group close to the surface of the silica is thought to allow water to penetrate and hydrate the silica, preventing self-association of the carbon chains.

Considerable chromatographic characterization has been given to alkyl amide bonded phases. Both monomeric and polymeric amide phases have been used to separate alkyl pyridine dicarboxylic acids [3] and indole carboxylic acids [4] using a water-acetonitrile (MeCN) mobile phase. Oligonucleotides have been separated on an alkyl amide stationary phase using a 90% buffer (pH = 5)-10% MeCN mobile phase [5]. At acidic pH values, the unamidated propyl amine groups can also cause the column to act as a weak anion exchanger. A C14-amide stationary phase end capped with di-isopropyl groups was found to not only be pH stable but highly effective for the separation of aromatic carboxylic acids and basic drugs [6]. Other commercial amide reversed phase columns for the separation of polar analytes have also been characterized [7].

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Most commercial HPLC column manufacturers sell an "aqueous" C18 column with a proprietary stationary phase primarily aimed for the separation of aliphatic polar organic acids using a 100% aqueous mobile phase. Sample mixtures analyzed such as fruit drinks, citrus extracts, and white wine often contain various subset combinations of oxalic, tartaric, malic, lactic, acetic, maleic, citric, succinic, and fumaric acid. Separation times of 10–20 min are the norm using a pH 2 dilute sulfuric acid or phosphate buffer. Comparison to a standard C18 column is sometimes made with invariably better analyte retention and separation on the polar group modified C18 column [8].

Surfactants have been commonly used as mobile phase modifiers for reversed phase HPLC, both in the sub-micellar and micellar modes, to facilitate the separation of polar, often charged organic compounds such as pharmaceuticals [9] and aromatic positional isomers [10,11]. We have been interested in using surfactant coated C18 columns to extend their utility, particularly if ultra-HPLC is of interest, due to the expense of such reversed phase columns. As compared to just using a C18 column, ion exclusion chromatography of organic acids on a sodium dodecyl sulfate (SDS) coated C18 column was effective for the analysis of soft drinks and fruit drinks [12]. Emphasis on the application of neutral surfactants as modifiers for HPLC has occurred quite recently [13]. Recently we have shown a Tween 20 coated C18 column can effectively separate positional aromatic carboxylic acid isomers, impurities often found in the synthesis of terephthalic acid [14].

Amino acid surfactants (AAS) are amphiphilic compounds in which the hydrophilic head is one amino acid unit or more [15,16]. AAS are considered as the most environmentally friendly type of surfactants due to their biodegradability and biocompatibility. AAS can be categorized according to the net charge on the surfactant molecules, exactly like classifying amino acids into cationic, anionic, zwitterionic, and neutral surfactants [17]. Many of these amino acid surfactants are used as antimicrobial and antifungal agents in cosmetics and food. Natural amino acids (except glycine), also present in AAS, are responsible for the formation of micelles that have chiral characteristics and optical activity [18–20]. The glutamic acid surfactant or *N*-lauroyl-L-glutamic acid (LLG) is one of the most synthesized of the AAS due to its broad applicability as a dispersing agent and emulsifier in facial soap, shampoos, cosmetics, and skincare products [21,22]. However, AAS have been used only as mobile phase additives in capillary electrophoresis [19] and micellar electrokinetic capillary chromatography [23], not in HPLC, to the best of our knowledge. In addition, reports of using any amino acid as a HPLC stationary phase is rare. One study immobilized a series of amino acids (Asp, Glu, Val, Pro, Hypro, Arg, Lys) separately on silica using 3-glycidoxypropyltriethoxysilane to form cation, anion, and zwitterion stationary phases However, little chromatographic application of these columns was shown, only separation of alkali and alkaline earth metal ions [24]. A glutamate derivative stationary phase, based on the reaction of γ-benzyl-L-glutamate-*N*-carboxyanhydride with aminopropyl silica, was utilized for the normal phase separation of polyaromatic hydrocarbons and phenols [25].

The aim of this study is to characterize a C18 stationary phase with a compact hydrophilic functional group at the end of the alkyl chain, not embedded near the silica surface, for the separation of polar analytes using a totally aqueous mobile phase. Our approach is to coat the C18 silica column with the LLG surfactant to form a stationary phase as shown in Fig. 1. The polar glutamate and amide group should keep the C18 chains apart allowing the polar analytes to penetrate permitting some hydrophobic retention as well as potentially hydrophilic interaction. Separation of ten aliphatic polar organic acids was possible with near baseline resolution in only 4 min using the LLG coated C18 column (3.5 μm particles) using a mobile phase of sulfuric acid (pH 1.8). Plots of ln retention factor (k) versus 1/temperature were linear indication one retention mechanism for these largely neutral organic acids was operative. Based on k versus alpha zero plots, the retention mechanism was considered primarily hydrophobic due to the increase in k as the fraction of the neutral analyte species increased. A comparison of the separation of the ten acids to a Tween 20 coated column was made to show the difference between a compact amino acid functional C18 end group and multiple long polyoxyethylene chains as the end group. Different fruit drink beverages and some vinegars (11 total) were analyzed for various subsets of these ten organic acid with malic and citric acids the most commonly found.

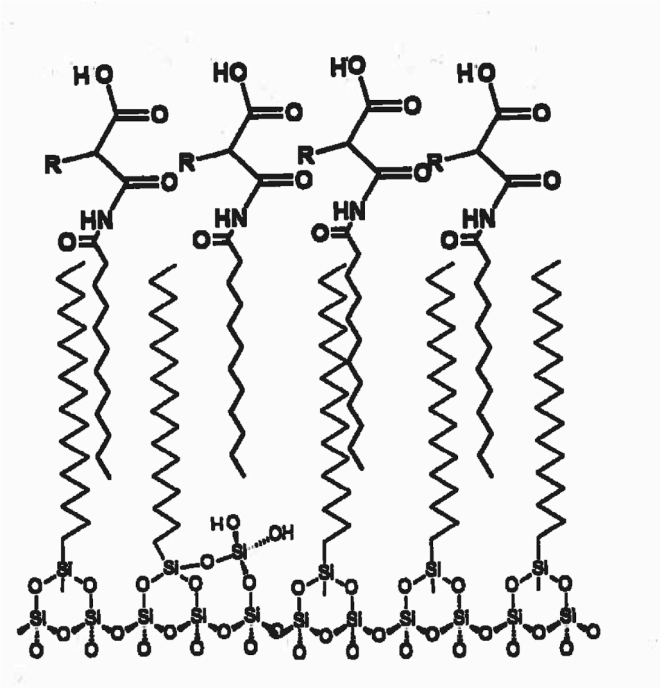


Fig. 1. The structure of *N*-lauroyl-L-glutamic acid (LLG) and its proposed interaction with the C18 stationary phase. R = CH_2–CH_2–COOH.

1.1. Instruments

All chromatographic analysis were done using an UltiMate 3000 UHPLC system (Thermo Fisher Scientific, Sunnyvale, CA, USA) equipped with a DGP 3600RS pump, online degasser, WPS 3000RS autosampler, TCC 3000RS temperature-controlled column compartment, and an Ultimate multiwavelength 3000RS UV-VIS detector. The UHPLC system was controlled by Chromeleon 7.2.1 software (Thermo Scientific, Sunnyvale, CA, USA). The separation was performed on a Waters Xbridge C18 column with dimensions of 150 mm × 4.6 mm, particle size 3.5 μm. The C18 chain is trifunctionally bonded to the end-capped silica particle providing pH stability between 1 and 12. The carbon load was 18% (ligand density 3.1 micromole/m^2), and the pore diameter is 135 Å with a surface area of 185 m^2/g.

1.2. Reagents

Sulfuric acid, purchased from Fisher Scientific (Pittsburgh, PA, USA), was diluted to the desired pH and used as the eluent in all separations. The lauroyl-L-glutamate (LLG) (96% pure) amino acid surfactant was generously provided by Ajinomoto Health & Nutrition North America, Inc. (Raleigh, NC). Sodium acetate (99.4% pure) and citric acid (> 99% pure) were purchased from Fisher Scientific (Pittsburgh, PA). Potassium fumarate (> 99% pure), DL-lactic acid (85% w/w syrup), maleic acid disodium salt (99% pure), L-malic acid, and L-tartaric acid (> 99.5% pure) were procured from Sigma-Aldrich (St. Louis, MO). Malonic acid was purchased from Matheson Coleman and Bell (Norwood, OH). Oxalic acid (> 99% pure) was from Allied Chemical and succinic acid was a gift. All carboxylic acid standard solutions were freshly prepared before each analysis.

1.3. Methods

All solutions in this work were made with deionized water purified using a Milli-Q (Millipore, Bedford, MA, USA) water purification system. The dynamic modification of the Xbridge C18 column was done using a 0.1% LLG solution prepared by dissolving 1 g of the solid reagent in 1 L of 100% aqueous solution brought to pH = 8.5 using dilute sodium hydroxide. The basic solution increased the solubility of the LLG and kept the "hydrophilic head" negatively charged to prevent its interaction with the C18 SP and ensure a laurate dynamic coating. The synthesized mobile phase was passed through the Xbridge C18 column for at least 5 h at a flow rate of 0.1 mL/min to ensure the saturation of the C18 stationary phase with the LLG surfactant. Then the column was washed for 30 min with 100% water to remove any unbonded surfactant. Chemical structures for all ten carboxylic acids (oxalic, tartaric, malic, malonic, lactic, acetic, maleic, citric, fumaric, and succinic) are shown in Fig. S1 and their acid dissociation constants (pK_A values) are listed in Table S1. Short-chain aliphatic acid mixtures including all ten acids were made for method development. Both column temperature and mobile phase pH were varied. UV-detection was normally maintained at 210 nm.

For the quantitative analysis, nine point calibration curves have been generated based on an average of triplicate measurements. Each carboxylic acid has a different molar absorptivity coefficient at 210 nm, which leads to use of different concentrations ranges to generate these calibration curves. The following is the concentration range used for each carboxylic acid: 1- oxalic (40–400 ppm), 2- tartaric (300–3000 ppm), 3-malic (400–4000 ppm), 4- malonic (500–5000 ppm), 5- lactic (500–5000 ppm), 6- acetic (500–5000 ppm), 7- maleic (18–180 ppm), 8- citric (500–5000 ppm), 9- fumaric (8–80 ppm), 10- succinic acid (500–5000 ppm). Ten real samples were chosen to be analyzed for their short chain carboxylic acid content. These commercial beverages are: regular Mountain Dew, diet Mountain Dew, distilled white vinegar (Kroger), apple cider vinegar (Kroger), organic apple cider vinegar (Simple Truth), black raspberry juice and peach (sparkling ice), healthy green juice (V8), organic concord juice (all from R.W. Knudsen), organic strawberry lemonade juice and organic apple juice, both from Santa Cruz. The carbonated beverages were sonicated for 20 min to remove the dissolved gas. All beverages were filtered through a 0.22 μm syringe filer before directly injected into the column without dilution.

2. Results and discussion

2.1. Optimization of chromatographic conditions

After the coating of the C18 stationary phase with LLG was achieved, only a 100% aqueous isocratic mobile phase was used for the chromatographic separations, giving this method advantages such as simplicity and low environmental impact. Initially, it was thought that this LLG coated C18 column would operate in the ion exclusion mode because the pKa1 and pKa2 values for the carboxyl groups of glutamic acid are 2.19 and 4.25, respectively. However, through use of Chemicalize software, the pKa1 and pKa2 values for LLG were predicated to be 3.62 and 4.39, respectively. This significant change in the pKa1 value is likely due to the electron withdrawing ability of the original amine group of glutamic acid being mitigated by its conversion to an amide group which tends to be electron donating, causing the COOH group to become less acidic. Therefore, an acidic mobile phase pH near 2.0, to ensure most of these organic acids are in their neutral form, will favor a hydrophobic interaction with the C18 and/or LLG alkyl amide chain and no repulsion from the neutral carboxyl groups. Although the amide group is somewhat zwitterionic in nature, ion exclusion has been ruled out as an important retention mechanism.

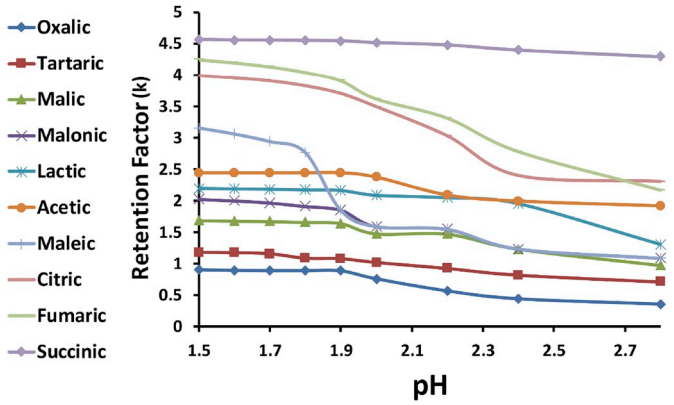


Fig. 2. The effect of changing pH on the retention factor k. The flow rate was 0.6 mL/min column temperature was set to 21 °C. Succinic (top trace, oxalic (bottom trace).

2.1.1. The effect of pH variation on the retention factor

The effect of changing the pH on the retention was thoroughly investigated between pH = 1.5 and pH = 2.8, as shown in Fig. 2. Oxalic acid is the least hydrophobic among the ten carboxylic acids due to the presence of two carboxylic groups with no C–H bonds in between. With a pKa_1 = 1.23, a significant fraction of oxalic acid will be negatively charged even at pH = 1.5 which makes it the least retained molecule among the mixture. At pH = 1.5, maleic acid (pKa1 = 1.83) has a significant fraction in its neutral form which makes its retention mechanism mainly dependent on hydrophobic interaction. By increasing the pH to almost 2.0, maleic acid dissociates to maleiate anion which is significantly decreased in retention due to its increased hydrophilic character. Citric acid and fumaric acid also see a marked decrease in retention at about pH 2.3 due to their respective low pKa1 values of 3.13 and 3.02. Other acids with low pKa1 values such as malonic and tartaric acid also show a decrease in retention with rising pH but it is not as pronounced due to their low k value range. Above pH = 2.1, most of the carboxylic acids are at least partially dissociated, resulting in their retention factor getting smaller by the increasing pH. Succinic acid is the least affected acid among the ten carboxylic acids under investigation due the fact that it has pKa_1 = 4.1; at pH = 2.8 or less, succinic acid will maintain its acid form.

The pH data, along with the pKa values, were used to calculate the alpha zero, the fraction of the organic acid in its neutral form. Plots of retention factor versus alpha zero are shown for all ten acids in Figs. S2 and S3 of the Supplementary Information. For the low pKa acids (oxalic and maleic), retention tends to level out around alpha zero values of 0.2 and 0.6, respectively. At lower pH values, it is assumed the retention would still increase. For five other acids (fumaric, citric, malonic, and tartaric, and malic), the retention steadily increased from alpha zero values in the 0.5–0.8 range to close to 1.0 where the k values leveled out. For the remaining three acids (lactic, acetic, and succinic) with the largest pKa1 values, alpha zero values started out high at least 0.9 and as expected, the increase in k was less marked but still levelling off near 1.0. These plots point to a hydrophbic retention mechanism.

2.1.2. The effect of column temperature

Retention factor (k) variation with respect to temperature from 30 °C to 9 °C was found for all the carboxylic acids under investigation (Fig. 3). As expected, lowering the temperature has an inverse influence on the retention factor; the lower the temperature, the longer retention. The baseline separation of all 10 acids was achieved above 20 °C; below 20 °C, the malonic acid peak merges with that of lactic acid and the citric acid peak merges with the fumaric acid peak. However, the elution sequence of the analytes off the column does not change over the studied temperature range of 20–30 °C.

Using the trends in Fig. 3, linear van't Hoff plots of ln k vs. 1/

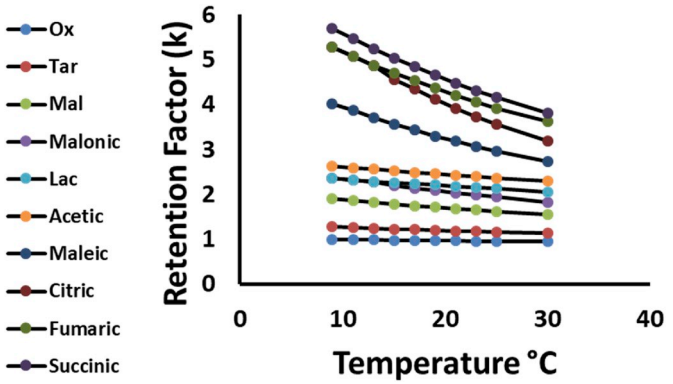


Fig. 3. The effect of changing the temperature on the retention factor k. The flow rate was 0.6 mL/min, and the mobile phase pH was 1.8. Oxalic (bottom trace), succinic (top trace).

Table 1
Coefficients a and b with correlation coefficient (R^2) for plots based on the van't Hoff equation: lnk' = a + b(1/T). Compounds listed in order of chromatographic retention.

Compound	a	b	R^2
Oxalic	−0.793	222	0.9928
Tartaric	−1.49	487	0.9983
Malic	−2.38	852	0.9992
Malonic	−2.90	1062	0.9948
Lactic	−1.02	529	0.9932
Acetic	−0.991	552	0.9996
Maleic	−4.27	1595	0.9996
Citric	−5.86	2125	0.9981
Fumaric	−3.83	1549	0.9998
Succinic	−4.11	1651	0.9998

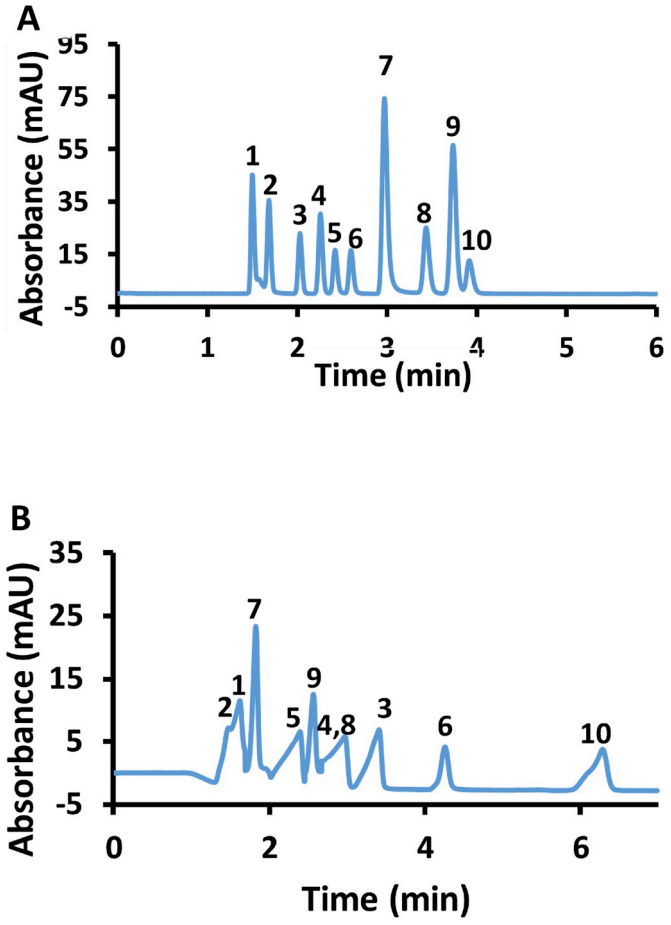


Fig. 4. A: Baseline separation of 10 acids using the Lauroyl L-Glutamate modified C18 column. Flow rate is 1.0 mL/min of 15% of 50 mM H_2SO_4 (pH = 1.8) at 23 °C. **B**: Ten acids chromatogram using C18 modified with Lauroyl L-Glutamate column. Flow rate is 0.6 mL/min of 0.5% of 50 mM H_2SO_4 (pH = 3.5) at 23 °C. Peak assignments for both chromatograms are: 1- Oxalic, 2- Tartaric, 3-Malic, 4- Malonic, 5- Lactic, 6- Acetic, 7- Maleic, 8- Citric, 9- Fumaric, 10- Succinic acid.

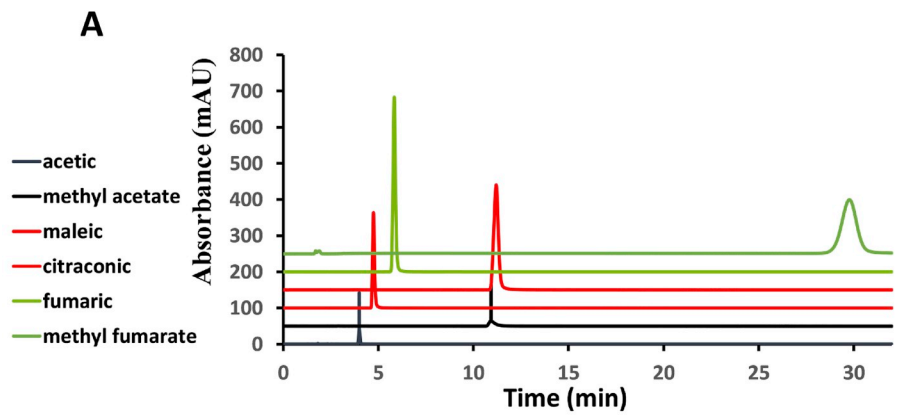
temperature (T) were generated for all ten acids. The intercept values, a measure of entropy, and the slope values, a measure of enthalpy, are summarized in Table 1. The data for oxalic and lactic acids (compounds with the lowest R^2 correlation coefficients) were also plotted as a second order polynomial equation. A comparison of the linear and quadratic plots is shown in Fig. S4 of the Supplementary Information. Although some curvature is evident, only a slight increase in the correlation coefficients were noted. The linearity of the van't Hoff plots points to the existence of one dominant retention mechanism, likely reversed phase. Due to the low pH of the mobile phase, all but oxalic and maleic acid are virtually completely in their neutral form. Apparently these two acids, despite being in two different charged states, are still undergoing retention by one mechanism [26].

2.1.3. Comparison of chromatograms to prior reports

As shown in Fig. 4A, all ten acids could be separated at the optimum temperature of 23 °C and a mobile phase pH of 1.8 with near baseline separation in just under 4 min. Although the hydrophilic part of the stationary phase is at the end of the alkyl chain, no evidence of phase collapse (very weak retention and poor peak resolution) was noted. The average column efficiency for the last two peaks in Fig. 4A was N = 15,000. Therefore a reduced plate height (h) of 2.9 can be calculated, not far off from the ideal minimum value of h = 2. This analysis time is considerably faster than the separation of a corresponding complex mixture on most commercial "aqueous" C18 columns. The retention order on our column is similar to that shown in a chromatogram done on such a column made by Dionex using a phosphate pH 2.7 mobile phase; this latter retention order was oxalic < tartaric < malic < lactic < acetic < citric < succinic < fumaric. In our previous study using a dodecyl sulfate coated C18 column (2.6 μm particles and 150 mm long) with a pH 2.4 mobile phase [12], resolution of all ten acids was not quite as good. The retention order of the organic acids was oxalic < tartaric < maleic = malonic < lactic < acetic < citric < succinic = fumaric which is again quite similar to what was found in this work even though both ion exclusion and hydrophobic retention mechanisms were deemed likely. Conversely, the separation in Fig. 4B, is unacceptable with broad peaks and lack of resolution, due likely to the change in mobile phase pH to 3.5. In general, the retention order is markedly different however maleic and fumaric acids still exhibit quite sharp peaks.

2.2. Retention mechanism study

Three methylated aliphatic carboxylic acids were chosen to compare their retention behavior with respect to the analogous unmethylated acids. Individual solutions of acetic acid/methyl acetate, fumaric acid/methyl fumarate, and maleic acid/citraconic acid were



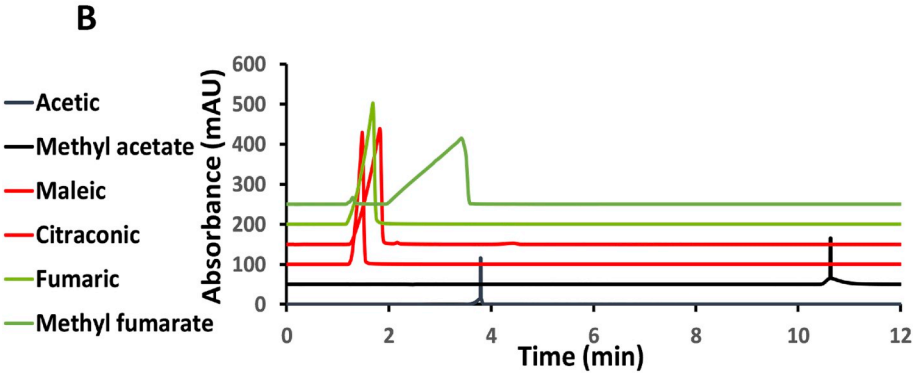


Fig. 5. A: Retention mechanism study for three selected acids and their methylated analogues at pH = 1.8. **B**: Retention mechanism study for same three acids and their analogues at pH = 4.2. Both chromatograms were done at 23 °C and 0.6 mL/min on LLG coated C18 column. Methyl fumarate (top trace), acetic acid (bottom trace).

chosen for this study. Citraconic acid is a methylated maleic acid through its aliphatic chain, which will leave both carboxylic groups untouched, with respective pKa1 and pKa2 values of 2.29 and 6.15. However, methyl acetate and methyl fumarate are methylated through their carboxyl group. Therefore methyl acetate is neutral, methyl fumarate is monoprotic, and citraconic acid is diprotic. As shown in Fig. 5, the chromatography comparison was done using mobile phases at pH = 1.8 and pH = 4.2. At pH = 1.8, the acids and their methylated derivatives (except maleic and citraconic acid) were primarily uncharged; the retention mainly depended on the hydrophobic interaction between these neutral molecules and the modified C18 stationary phase (Fig. 5A). Retention times of methyl acetate, citraconic acid, and particularly methyl fumarate are respectively longer than those of acetic, maleic, and fumaric acid at this low pH. At pH = 4.2, acetic acid and methyl acetate (neutral molecules) are almost unaffected by this change in pH (Fig. 5B). The other compounds are only weakly retained due to their ionization of the carboxylic acid groups. Favored retention of the methylated compounds again indicates the importance of the hydrophobic retention mechanism.

A comparison study was done by changing the anionic LLG surfactant on the column to the non-ionic Tween 20 surfactant to show the effect of the surfactant on the retention mechanism. The dynamic modification of the same C18 stationary phase with Tween 20 was done using the same conditions used previously with LLG. Fig. 6 shows the separation of the same standard set of organic acids used in Fig. 4A. Lack of peak resolution is evident with significant overlap between oxalic/malic acid and citric/succinic peak pairs. The retention order is roughly the same with the exception of maleic acid being retained the longest. Analysis time is about 1.5 times longer for the Tween chromatogram as compared to the LLG chromatogram indicating analyte mass transfer has become not as favored. The Tween 20 surfactant with

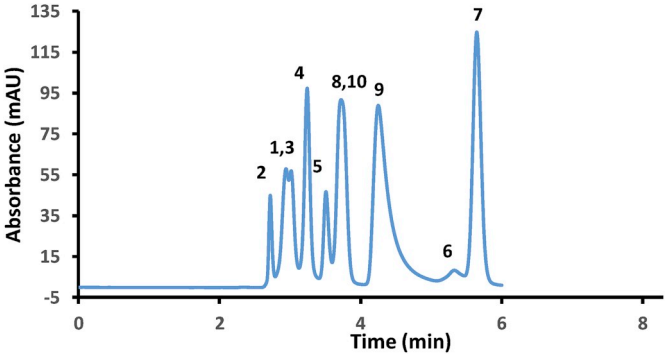


Fig. 6. Chromatogram of ten acids using a Tween 20 coated C18 column. Flow rate 1.0 mL/min of 15% of 50 mM H_2SO_4 at 23 °C. 1- Oxalic 2- Tartaric 3-Malic 4- Malonic 5- Lactic 6- Acetic 7- Maleic 8- Citric 9- Fumaric 10- Succinic acid.

four poly(ethyleneglycol) (PEG) chains is much bulkier in terms of the hydrophilic end group as compared to LLG. Although the PEG chains would be accessible for modest hydrophobic analyte interaction, penetration of the organic acids to the C18 and/or Tween alkyl chain would likely be more difficult due to steric reasons. Further comparison work with different Tween and amino acid surfactants is warranted.

2.3. Analytical figures of merit and quantitative analysis

A variety of samples (eleven total) encompassing soft drinks, vinegars, and juices (as indicated in the experimental section) were analyzed for their aliphatic acid content using the external standard method. Nine point calibration curves were generated using an average of triplicate measurements. Because each carboxylic acid has a different

Table 2
Limits of detections, limits of quantitation and regression parameters of the ten calibration curves.

Compound	A	B	R^2	LOD	LOQ
Oxalic	−0.037	1.062	0.9973	0.033(0.594)	0.4869
Tartaric	0.1076	0.24	0.996	0.146(4.382)	1.060
Malic	0.0491	0.11	0.996	0.318(8.519)	1.253
Malonic	0.0858	0.093	0.9948	0.376(7.820)	2.556
Lactic	0.0447	0.046	0.9966	0.7667(13.8)	3.120
Acetic	0.0524	0.037	0.9979	0.9359(11.23)	0.5604
Maleic	0.1804	9.946	0.9935	0.0035(0.082)	0.0117
Citric	0.0829	0.208	0.9976	0.1681(6.456)	1.572
Fumaric	0.1672	17.97	0.9977	0.002(0.045)	0.0065
Succinic	0.0035	0.074	0.9957	0.4717(11.13)	0.4869

Column A is y-intercept in nanomole; column B is the slope of the calibration curve.
R^2 is the correlation coefficient.
The limit of detection (LOD) values are given in nanomoles (and in ppm based on a 5 μL injection volume). Their values were calculated using the following equation: LOD = (3*σ)/B where σ is the standard deviation of the y-intercept. The limit of quantitation (LOQ) values are given in nanomoles. Their values were calculated using the following equation: LOD = (10*σ)/B where σ is the standard deviation of the y-intercept.

absorptivity coefficient, the concentration range was different for each acid. Table 2 summarizes the analytical figures of merit. Detection limits are most favorable for maleic and fumaric acids, likely because of the carbon-carbon double bond increasing the molar absorptivity. Fig. 7 shows four chromatograms of: a standard mixture of ten carboxylic acids (**7A**), black raspberry juice (Sparkling Ice) (**7B**). Peach nectarine juice (Sparkling Ice) (**7C**), organic apple cider vinegar (Simple Truth) (**7D**). The four samples of black raspberry (Sparkling Ice), peach nectarine (Sparkling Ice), healthy green (V8), and organic apple juice (Santa Cruz) showed a peak at 4.52 min. This unknown compound has an absorbance peak around 260 nm, which suggests the presence of ascorbic acid. Although we have done multiwavelength detection to check for possible interfering sample components (only ascorbic acid was noted), we cannot positively rule this issue out. The most common aliphatic carboxylic acid in the natural juices and vinegars (except distilled) was malic acid found in the 500–6000 ppm range; acids not found were oxalic, malonic, and maleic (Table 3). Fumaric acid was also commonly found in the 1–65 ppm range in most of the juice samples as was citric acid in the 10–6000 ppm range. Acetic acid had very similar concentrations in the three vinegar samples which were all close to the label value. Relative standard deviation for the organic acids detected in the apple cider vinegar and the sparkling ice black raspberry ranged from 0.3 to 4.3% with the exception of citric acid at 7.8%.

3. Conclusions

To the best of our knowledge, an amino acid surfactant coated on a C18 column was used for the first time as a stationary phase compatible with a totally aqueous mobile phase. Although the design of this stationary phase, with the hydrophilic group of glutamic acid at the end of the alkyl chain, is in contrast to such columns designed for a totally aqueous mobile phase that have a polar (often amide) group embedded near the silica surface, no evidence of phase collapse was noted. After optimizing chromatographic conditions such as pH to 1.8 and temperature to 23 °C, near baseline resolution of the ten carboxylic acids within 4 min with excellent peak shape was possible. Linear relationships of ln retention factor (k) versus 1/Temperature (T) (van't Hoff plots) were generated for all the acids indicating a single retention mechanism was likely. As the pH of the mobile phase decreased, the analyte retention factors increased due to the increase of the fraction of the analyte with neutral charge (alpha zero). The surfactant amide linkage, being electron donating, increased the pKa of the more acidic carboxyl group of glutamic acid so both carboxyl groups were protonated (neutral) at pH 1.8. The exact nature of the retention mechanism is uncertain but there certainly seems to be a pronounced hydrophobic component as additionally evident by the large difference in retention of fumaric acid and methyl fumarate at pH 1.8. In addition, eleven beverage samples were analyzed for their aliphatic carboxylic acid contents showing that malic, fumaric, and citric acids were the most common constituents. Additional amino acid surfactants based on glycine, alanine, and lysine are commercially available which could establish an entire new class of HPLC columns for separation of polar organic compounds under totally aqueous conditions.

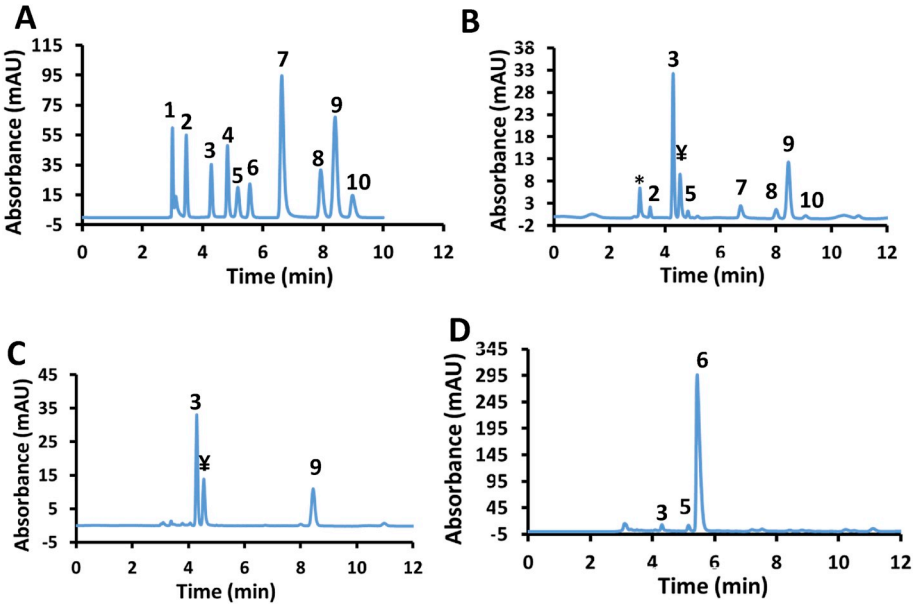


Fig. 7. A: Chromatogram of a standard mixture of ten aliphatic carboxylic acids. Flow rate is 0.5 mL/min of 15% of 50 mM H_2SO_4 (pH = 1.8) at 23 °C. **B:** Chromatogram of black raspberry juice (Sparkling Ice). **C:** Chromatogram of peach nectarine juice (Sparkling Ice). **D:** Chromatogram of organic apple cider vinegar (Simple Truth). 1- Oxalic, 2- Tartaric, 3-Malic, 4- Malonic, 5- Lactic, 6- Acetic, 7- Maleic, 8- Citric, 9- Fumaric, 10- Succinic acid, * unknown, ¥ ascorbic acid.

Table 3
List of the beverages analyzed for their carboxylic acid contents (ppm values).

	Mountain Dew	Mountain Dew diet	**Kroger distilled white vinegar**	**Kroger apple cider vinegar**	Simple truth organic vinegar	Sparkling ice black raspberry	Sparkling ice peach nectarine	**V8 healthy green**	**R.W. Knudsen organic concord**	**Santa Cruz strawberry lemonade**	**Santa Cruz apple organic**
Oxalic	-	-	-	-	-	-	-	-	-	-	-
Tartaric	-	-	-	**53.71**	-	**44.34**	-	-	**7100**	-	**26.76**
Malic	-	-	-	**992.5**	**1262**	**2900**	**2976**	**3283**	**5598**	**511.3**	**6432**
Malonic	-	-	-	-	-	-	-	-	-	-	-
Lactic	-	-	-	**1183**	**1784**	-	-	-	-	-	-
Acetic	-	-	**6174**	**6141**	**6256**	-	-	-	-	-	-
Maleic	-	-	-	-	-	-	-	-	-	-	-
Citric	**1876**	**2732**	-	-	-	**204.1**	**10.3**	**3900**	**260**	**6543**	-
Fumaric	-	-	-	-	**1.05**	**11.18**	**9.63**	**28.01**	**63.8**		**0.624**
Succinic	-	-	-	-	-	**168.8**	-	-	-	-	-

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.120807.

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