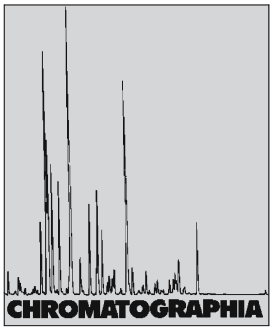
Investigating the Effect of Chromatographic Conditions on Retention of Organic Acids in Hydrophilic Interaction Chromatography Using a Design of Experiment



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Abstract

Small organic acids have shown significant retention on various stationary phases, such as amide, amino, aspartamide, silica and sulfobetaine phase commonly used in hydrophilic interaction chromatography (HILIC). This study investigated the effect of chromatographic conditions on the retention behavior of organic acids in HILIC using the tool of design of experiment (DOE). The results of the DOE study indicated that both the content of organic solvent (i.e., acetonitrile) and salt concentration in the mobile phase had significant effects on the retention of organic acids. Higher content of organic solvent in the mobile phase led to a significant increase in retention on all types of stationary phases. Increasing salt concentration also resulted in a moderate increase in retention; however, the effect of salt concentration varied with the type of stationary phase. The study also revealed that column temperature had less impact on retention than organic solvent content and salt concentration in HILIC.

Keywords

Hydrophilic interaction chromatography
Design of experiment
Organic acid

Introduction

Hydrophilic interaction chromatography (HILIC) as a viable separation technique has found an increasing number of applications in analyzing a wide variety of polar compounds in various matrixes, such as foods, drugs and biological fluids [1–11]. Small organic acids are one group of compounds that have shown suitable for HILIC separation [12–16]. Ion chromatography (IC) has been conventionally used to analyze organic acids; however, the mobile phase condition in IC is not amiable to mass spectrometry (MS), which makes it more difficult to perform MS analysis on organic acids when needed. In contrast, the mobile phase with a high content of organic solvents and volatile salt at moderate concentrations not only renders coupling HILIC to MS very easy, but also significantly boosts MS sensitivity for organic acids [17]. Despite many applications in the literature, there have been fewer systematic studies on the effects of chromatographic conditions such as stationary phase, solvent content, salt concentration, and column temperature on the retention behavior of organic acids in HILIC.

A study aimed at understanding the effect of various chromatographic parameters normally involves a fairly large number of experiments covering a reasonable range of each parameter. If investigating one parameter at a time, it would take quite a long time to complete the study, and might not reveal the inter-relationship among various factors if not properly designed. Design of experiment (DOE) is a structured and organized method based on statistical principles to explore the boundaries and relationships of variables involved in a certain process [18]. In the field of separation science, DOE principles have been applied to optimizing separation conditions and validating chromatographic methods [19–22]. In this study, DOE was used to study the effect of various chromatographic conditions on the retention of organic acids in HILIC. The use of DOE principles allowed a simultaneous study of multiple variables (e.g., acetonitrile content, salt concentration and column temperature), which led to a significant decrease in the number of necessary experiments. This not only helped to shorten the time needed to complete the study but also provided a better understanding of the relative importance and inter-relationship of the experimental variables.

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Table 1. Retention and selectivity factors of the stationary phases for organic acids

	Retention factor (k')/Selectivity factor (α)				
	Amide phase	Aspartamide phase	Silica phase	Sulfobetaine phase	Amino phase
Salicylic acid	1.47/0.66	1.55/0.55	1.29/0.61	1.30/0.76	1.73/0.45
Gentisic acid	1.95/0.88	2.23/0.80	1.39/0.65	2.10/1.23	2.28/0.60
Acetylsalicylic acid	2.21/1.00	2.80/1.00	2.12/1.00	1.71/1.00	3.82/1.00
Salicyluric acid	2.69/1.21	3.53/1.26	2.17/1.02	1.95/1.14	4.81/1.26
Hippuric acid	3.50/1.58	4.35/1.55	2.87/1.35	2.26/1.32	5.93/1.55
α-Hydroxyhippuric acid	3.70/1.67	5.04/1.80	2.29/1.08	2.26/1.32	5.20/1.36

Experimental conditions are the same as in Fig. 2

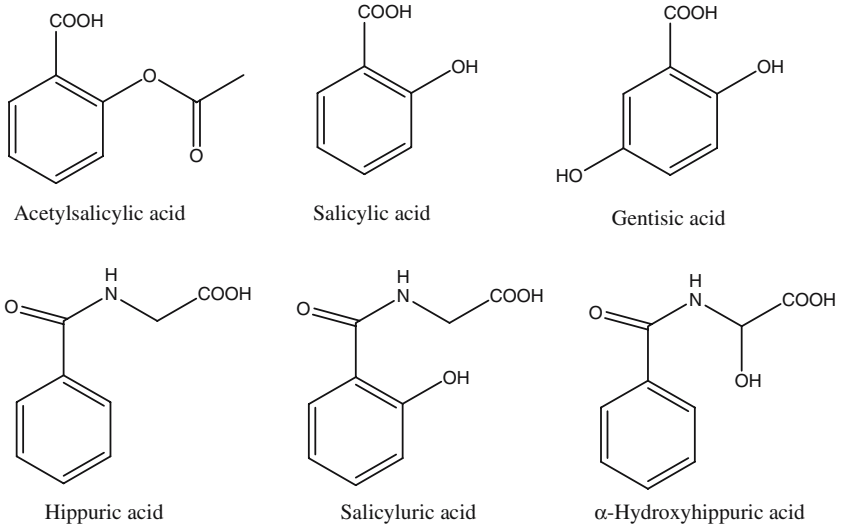

Fig. 1. The structures of organic acids

Experimental

Reagents

HPLC grade acetonitrile (ACN) was purchased from EM Science (Hawthorne, NY, USA), and HPLC grade water was obtained from a Millipore Milli-Q Gradient purification system (Bedford, MA, USA). Ammonium acetate was of ACS grade from Aldrich (Milwaukee, WI, USA). Salicylic acid, acetylsalicylic acid, salicyluric acid, gentisic acid, hippuric acid, and α-hydroxyhippuric acid were also purchased from Aldrich. YMC-Pack NH_2 (5 µm, 4.6 × 250 mm, pore size 120 angstroms) and Atlantis HILIC Silica (5 µm, 4.6 × 250 mm, pore size 135 angstroms) columns were obtained from Waters (Milford, MA, USA), TSKgel Amide-80 column (5 µm, 4.6 × 250 mm, pore size 80 angstroms) from Tosoh Bioscience (Montgomeryville, PA USA), and PolyHydroxyethyl A (5 µm, 4.6 × 200 mm, pore size 100 angstroms) and ZIC-HILIC (5 µm, 4.6 × 250 mm, pore size 200 angstroms) columns from The Nest Group (Southborough, MA, USA). All columns were equilibrated with the mobile phase prior to use and washed with acetonitrile and water (60/40, *v*/*v*) after use.

Instrumentation

All experiments were conducted on a HP1100 HPLC system (Agilent Technologies, Palo Alto, CA, USA) consisting of a quaternary pump, a degasser, a column heater and an auto-injector. The HPLC system was equipped with a diode-array detector (DAD). Agilent ChemStation software (Rev. A.09.01) was used for data acquisition and analysis.

Mobile phase was prepared by mixing appropriate volumes of acetonitrile with water and stock ammonium acetate solution (~100 mM) to reach the desired ACN content and salt concentration. The stock ammonium acetate solution was prepared by dissolving an appropriate amount of ammonium acetate in water. The pH of the stock ammonium acetate solution was not adjusted before mixing with ACN and water. The mobile phase was not pre-heated prior to entering the column when the column was operated at elevated temperatures.

Data Analysis

The experimental design was generated by a DOE software, Fusion Pro™ obtained from S-Matrix Corporation (Eureka, CA, USA). Raw chromatographic data (i.e., retention time) were entered into the DOE software for statistical analysis.

Results and Discussion

Retention and Selectivity on HILIC Stationary Phases

Six organic acids were selected as model compounds for this study, including acetylsalicylic acid, salicylic acid, salicyluric acid, gentisic acid, hippuric acid and α-hydroxyhippuric acid, which have similar structures as shown in Fig. 1. Salicylic acid, gentisic acid and salicyluric acid are known metabolites of acetylsalicylic acid, and α-hydroxyhippuric acid is an isomer of salicyluric acid. To understand the stationary phase effect, five columns representing the most common stationary phases used in HILIC were investigated for the separation of selected organic acids. The HILIC stationary phases have different functional groups and surface charge characteristics [16]. In the TSKgel Amide-80 column, ionization of residual surface silanol groups at pH above four imparts negative charges to the column even though the amide ligands are neutral. In the PolyHydroxyethyl A column, the polypeptide coating can be either positively charged at a pH below 4.4 or negatively charged at a pH above 4.4 presumably due to the presence of free N- and C-termini [23]. In the ZIC-HILIC column, the sulfonate groups on the outside of the sulfobetaine ligands give the column cation-exchange properties despite of the overall zwitterionic nature of the ligand. The silica phase in HILIC Silica column is negatively charged due to silanol deprotonation above pH 4, and the amino phase in YMC-Pack NH_2 column, in contrast, is

positively charged under the current experimental conditions. The selected organic acids were separated on the five stationary phases under the same experimental conditions, as shown in Fig. 2. To quantitatively compare stationary phase selectivity, selectivity factors (α) were calculated against acetylsalicylic acid, since it is the parent drug in aspirin metabolite analysis. Table 1 presents the retention and selectivity factors of six acids on the stationary phases.

As shown in Fig. 2a, b, baseline separation of all organic acids was achieved on the amide and aspartamide phases with good peak shape, however a small tailing was observed for hippuric acid on both phases. Capacity factor data (Table 1) indicated that the aspartamide phase had stronger retention for the acids than the amide phase, but both phases exhibited very similar selectivity towards most acids. The aspartamide phase showed a slightly higher selectivity towards α-hydroxyhippuric acid than the amide phase. In comparison, the silica and sulfobetaine phases had much less retention for most of the acids and also showed a different selectivity than the amide and aspartamide phases. The stronger retention on the amide and aspartamide phases could be attributed to thicker and more hydrated layer of the coating material according to Alpert's partitioning model [24]. Of course, the retention on the silica and sulfobetaine phases could also be reduced by electrostatic repulsion of the negatively charged acids from the negatively charged silica and sulfobetaine surfaces. In addition, it was also interesting to compare the selectivity of the silica and sulfobetaine phases (Fig. 2c, d). For example, gentisic acid was least well retained by the silica phase, but was retained very strongly on the sulfobetaine phase. Acetylsalicylic acid and salicyluric acid were barely resolved on the silica phase, but were baseline separated on the sulfobetaine phase. Similarly, hippuric acid and α-hydroxyhippuric acid co-eluted on the sulfobetaine phase, but were well separated on the silica phase. In comparison, the amino phase had the strongest retention for the acids, but was less selective towards salicylic acid and gentisic acid compared to phases other than silica. The peaks in Fig. 2e were broad and even distorted in some cases. The strong retention and peak broadening might be the result of mixed-mode separation on the amino phase, which will be discussed later.

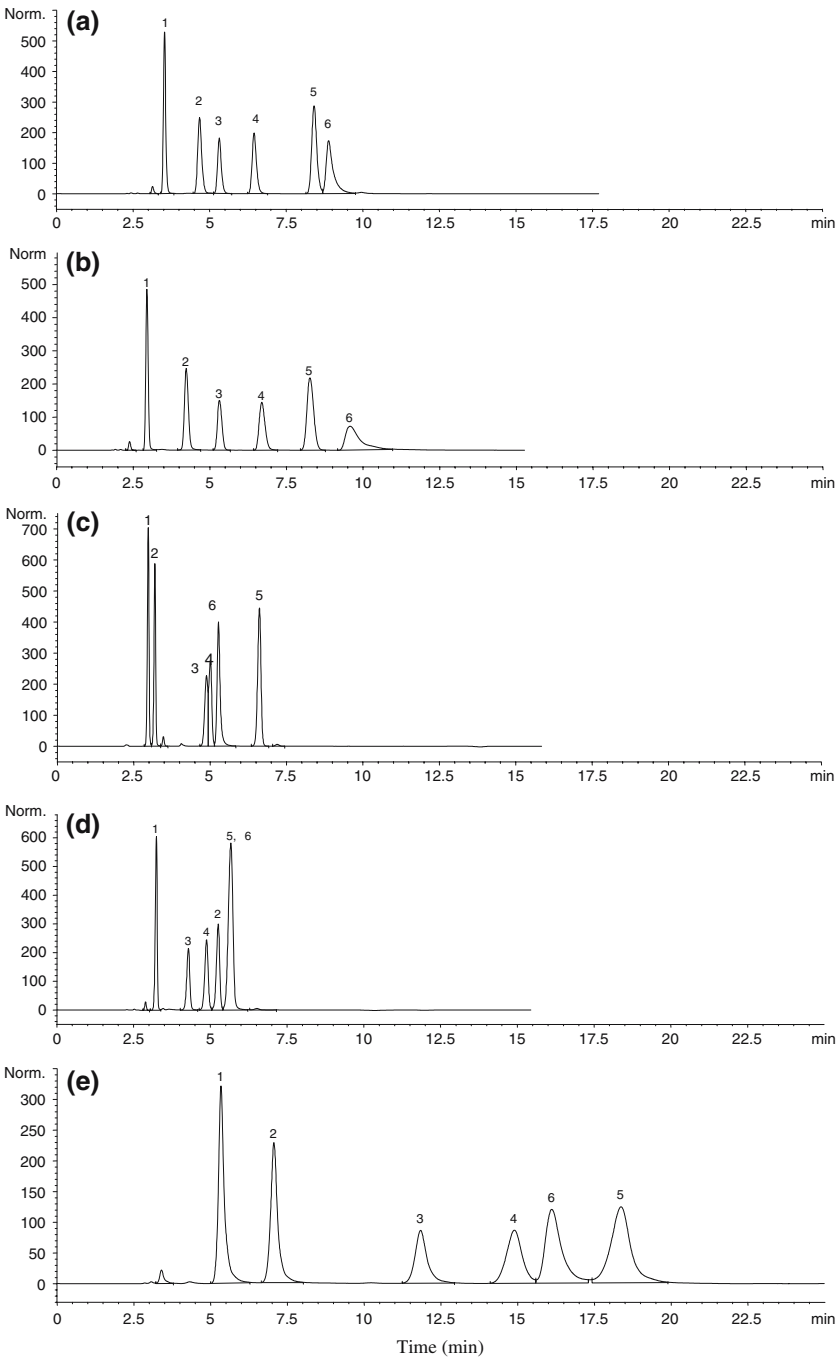

Fig. 2. Chromatograms for acid separation on (**a**) TSK-gel Amide-80 column, (**b**) Polyhydroxyethyl A column, (**c**) HILIC Silica column, (**d**) ZIC-HILIC column and (**e**) YMC Pack NH_2 column. Mobile phase: acetonitrile/water (85/15, *v*/*v*) containing 10 mM ammonium acetate. Flow rate 1 mL min^{-1}, column temperature 30 °C, and UV detection at 228 nm. Peaks **1** = salicylic acid, **2** = gentisic acid, **3** = acetylsalicylic acid, **4** = salicyluric acid, **5** = hippuric acid, **6** = α-hydroxyhippuric acid

Experimental Design

In addition to the stationary phase, other chromatographic conditions (e.g., solvent content, salt concentration and column temperature) also have significant effects on the retention and selectivity for organic acids [16]. The conventional approach to this type of study is to vary one parameter at a time while keeping others unchanged. If done systematically, it would take a fairly large number of experiments and a long time to complete

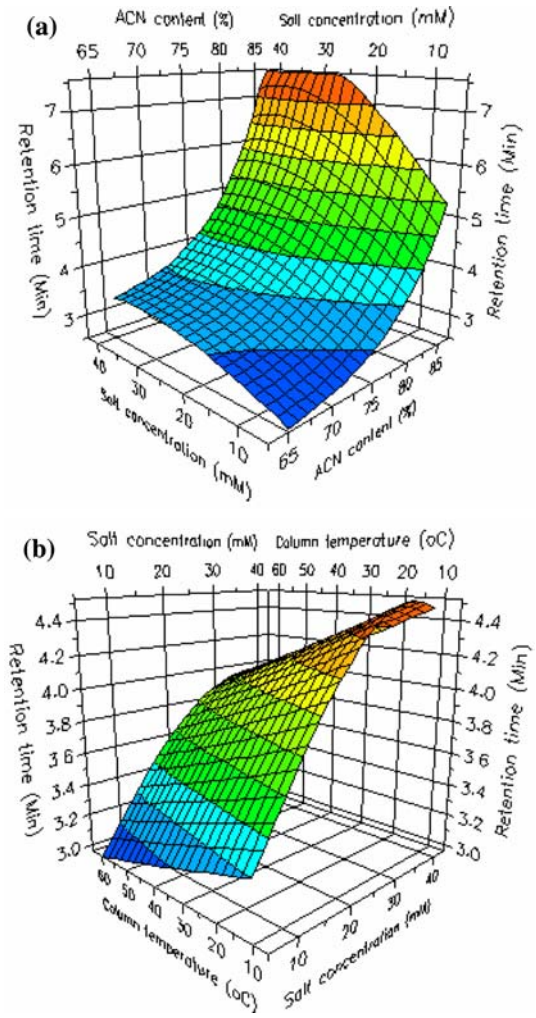

Fig. 3. Response surface for the amide phase (TSK-gel Amide-80 column)

Table 2. Experimental design

Run no.	Acetonitrile content (%)	Salt concentration (mM)	Column temperature (°C)
1	85	5	35
2	70	31	23
3	65	40	10
4	75	5	60
5	80	14	48
6	75	23	35
7	65	40	10
8	70	31	48
9	65	5	35
10	80	31	23
11	85	40	60
12	65	23	60
13	85	40	10
14	75	23	35
15	85	5	10
16	80	31	48
17	65	40	60
18	75	5	60
19	85	23	60
20	65	5	10

such a study. For example, it would need 125 (5^3) experiments to study 3 parameters at 5 levels. Even if all 125 experiments were completed, the interrelationship among the parameters might not be clearly revealed by this approach. This study employed experimental design to investigate the effect of chromatographic conditions on the separation of organic acids. Previous studies have identified organic solvent content, salt concentration in the mobile phase and column temperature as the most important parameters to HILIC separation [16]. In this study, acetonitrile was used as the organic solvent in the mobile phase with a range of 65–85% (*v*/*v*), and the concentration of ammonium acetate ranged from 5 to 40 mM. The upper level of acetonitrile content and ammonium acetate concentration were limited due to the low solubility of ammonium acetate. In addition, the column temperature was varied from 10 to 60 °C. In order to investigate three parameters (i.e., solvent content, salt concentration and column temperature) at 5 levels, this study employed a 3 × 5 design with a center. The experimental design in Table 2 lists 20 experiments including 2 dummies (No. 14 and 18). Two other experiments (No. 11 and 13) were not conducted because 40 mM was above the solubility limit of ammonium acetate in the mobile phase containing 85% ACN. This experimental limitation had only minor impact on data analysis at high ACN and salt conditions.

Effect of Experimental Conditions

The experimental design was applied to each of the five stationary phases in this study. The experimental data (i.e., retention time) for each acid was then entered into the DOE software for statistical analysis. To illustrate the effects of experimental conditions on acid retention, 3D response surfaces as shown in Figs. 3, 4, and 5 were generated for each phase, which depicted the relationship between retention time and experimental variables. Since all the acids had very similar response, only the response surfaces for salicyluric acid are presented for further discussion. For example, Fig. 3a, b show the effects of ACN content, salt concentration and column temperature on retention time on the amide phase. The retention time of salicyluric acid not only increased significantly with the ACN content, but also increased moderately with the salt concentration as shown in Fig. 3a. The plateau in Fig. 3a was due to the lack of data at high salt concentration and high ACN content in the mobile phase (40 mM ammonium acetate in 85% ACN). The response surface for column temperature and salt concentration in Fig. 3b shows that the retention time decreased as the column temperature increased, and there seemed to be a stronger temperature effect at higher salt concentration than at low concentration. Similar response surfaces were also observed on the aspartamide and silica phases (not shown). Small differences in surface response might reflect the difference in stationary phase chemistry. For example, a slight curvature was observed in the temperature/salt concentration

response surface for the sulfobetaine phase, as shown in Fig. 4b.

Interestingly, the amino phase had very different response surfaces as shown in Fig. 5a, b. The retention time showed a drastic decrease as the salt concentration increased, in direct contrast to the observations with the other phases. This is possibly due to the electrostatic interactions between the positively charged amino phase and negatively charged acids under experimental conditions. Increasing salt concentration diminished the ion-exchange effect, resulting in reduced retention. With the electrostatic interaction with the amino phase reduced at higher salt concentrations (> 20 mM), the retention showed a similar increase with the ACN content effect, indicating the role of hydrophilic interaction in retention. Therefore, both the hydrophilic and electrostatic interactions might have contributed to the retention of the organic acids at lower salt concentration and relatively high ACN content (> 75%). This study provided strong evidence that the separation of organic acids on the amino phase might be based on a mixed-mode mechanism, which explains the long retention time and imperfect peak shape observed with the amino phase as shown in Fig. 2.

In addition, statistical analysis using the DOE software also provided a means to understand the relative importance of the experimental variables (i.e., ACN content, salt concentration and column temperature) for each stationary phase. In model term ranking, the variable that has the most significant impact is assigned a ranking of 1, and other variables are ranked based on the experimental data. Figure 6 shows the model term ranking chart for salicyluric acid on the five stationary phases used in this study. As expected, the ACN content was the most significant factor in determining retention on the amide, aspartamide, silica and sulfobetaine phases. On the amino phase, however, the salt concentration ranked even higher than the ACN content, indicating that ion-exchange was the predominant mechanism in the separation of organic acids. In addition, it is also interesting to note the relative importance of the salt concentration on different types of the stationary phase. The ranking in Fig. 6 indicates that the salt concentration had more significant effect on retention on the charged stationary phases. A very strong salt effect was observed on the silica and sulfobetaine phases, indicating that the electrostatic effect was rather significant as the result of the interactions between the negatively charged stationary phase and organic acids. This was consistent with reduced retention observed on the silica and sulfobetaine phases compared to the amide and aspartamide phases. In comparison, the salt concentration had a smaller impact on retention on the non-charged phases, especially the aspartamide phase.

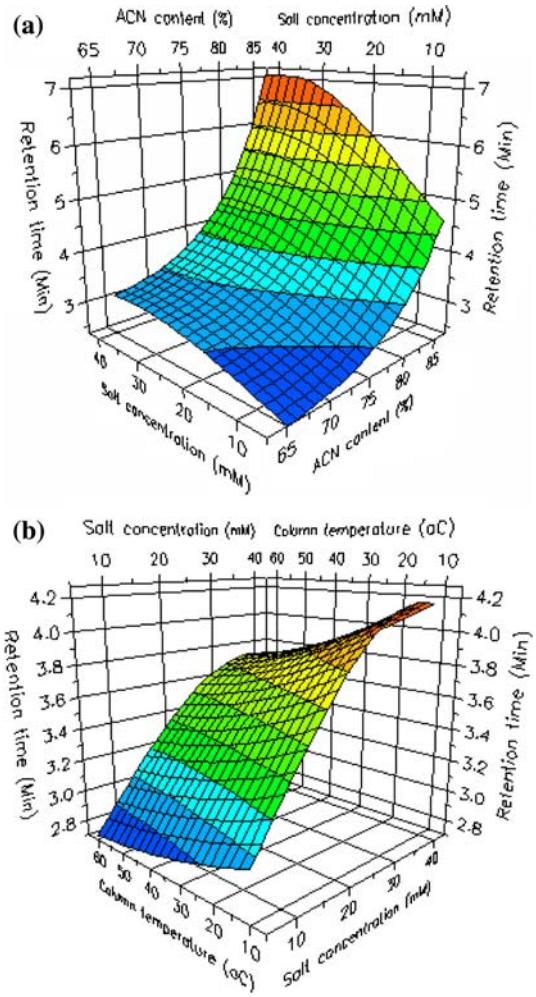

Fig. 4. Response surface for the aspartamide phase (Polyhydroxyethyl A column)

Effect of Salt Concentration

The DOE study clearly indicated from a statistical perspective that the salt concentration had a significant, but moderate effect on the retention of organic acids on the HILIC stationary phases. This finding was further confirmed by experimental results as shown in Fig. 7. The retention factor of salicyluric acid was obtained on four stationary phases (i.e., amide, aspartamide, silica and sulfobetaine phases) as the salt concentration was varied from 5 to 40 mM while other conditions (i.e., acetonitrile content and temperature) remained constant (80% ACN and 30 °C). The retention factor of salicyluric acid increased significantly on all four stationary phases when the salt concentration was increased from 5 to 15 mM, but gradually leveled off upon further increasing the salt concentration to 40 mM except on the silica phase. Similar trend was also observed for acidic compounds under HILIC conditions [25]. As discussed in the previous section, all the stationary phases under investigation carried different levels of negative charges from either ionized silanol groups (silica and amide phases), deprotonated carboxylic groups (aspartamide phase), or sulfate groups (sulfobetaine phase), which led to the

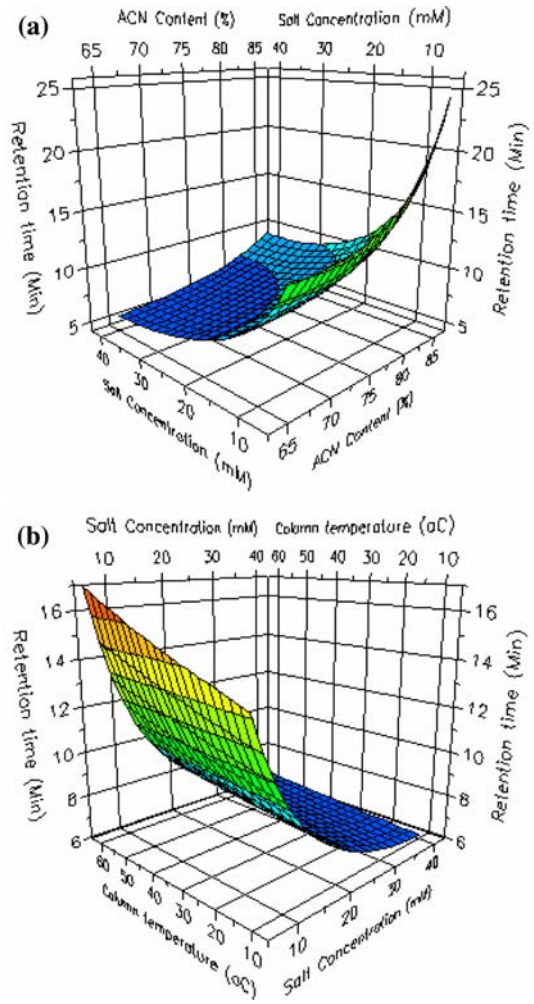

Fig. 5. Response surface for the amino phase (YMC-Pack NH_2 column)

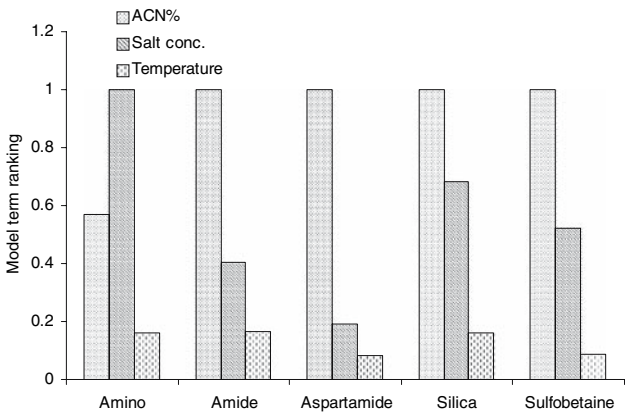

Fig. 6. Model term ranking chart for the HILIC stationary phases

electrostatic repulsion to the negatively charged organic acids under the experimental conditions. An increase in salt concentration could reduce the electrostatic repulsion, thus resulting in stronger retention.

Even though the amide and aspartamide phases showed similar selectivity towards the organic acids (Fig. 2), the salt concentration may have a slightly different effect on the two phases as indicated in Fig. 7. This may be related to the difference in the amount of negative charges present at the coating surface. To further understand the effect of salt concentration on the two phases, the retention behavior of salicyluric acid was studied in the temperature range of 10–60 °C using a mobile phase containing 80% acetonitrile at different ammonium acetate concentrations (15, 25 and 40 mM). The logarithm of retention factor (k') was plotted against 1/T as shown in Fig. 8. On the amide phase, salicyluric acid displayed nearly perfect linear behavior with regression coefficient (R^2) ranging from 0.992 to 0.999. A slight deviation at 40 mM was observed at higher temperature (R^2–0.992). In comparison, an obvious deviation from linearity was observed on the aspartamide phase at all concentrations with R^2 ranging from 0.943 to 0.954. The deviation of the van't Hoff plots from linearity usually indicates changes in the retention mechanism [26]. Even though the electrostatic interaction might be minimized at salt concentration above 15 mM as shown in Fig. 7, the complicated surface chemistry of the polyaspartamide coating might have provided sites for specific interactions (e.g., hydrogen bonding) in addition to hydrophilic interaction.

Conclusion

As in reversed-phase liquid chromatography, the stationary phase has shown to play a very important role in determining the retention and selectivity for organic acids in HILIC. The aspartamide phase exhibited stronger retention for the acids than other phases (except the amino phase), but had similar selectivity to the amide phase. The silica and sulfobetaine phases displayed reduced retention for the acids possibly due to electrostatic repulsion from the negatively charged groups on the stationary phase; however, the two phases showed almost complementary selectivity towards the acids. The strong salt effect on the amino phase revealed by the DOE study indicated that the acid separation on the amino phase was predominantly based on electrostatic effects, rather than hydrophilic partitioning. The DOE results not only confirmed that the organic content was the most significant factor in determining the retention in HILIC (with the exception of the amino phase), but also revealed an interesting effect of

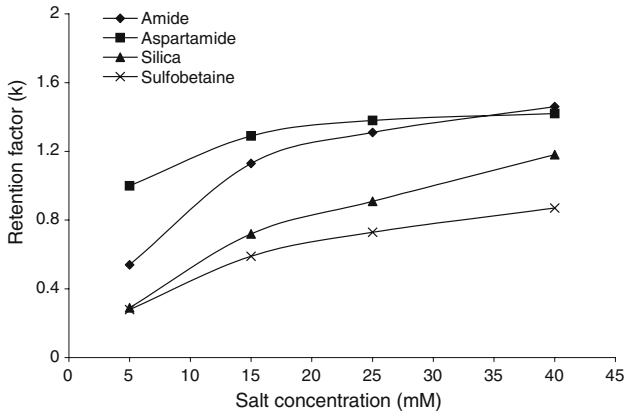

Fig. 7. Plots of retention factor (k') versus salt concentration for salicyluric acid on four HILIC stationary phases. Mobile phase: acetonitrile/water (80/20, v/v) containing 5–40 mM ammonium acetate. Flow rate 1 mL min^{-1}, column temperature 30 °C, and UV detection at 228 nm

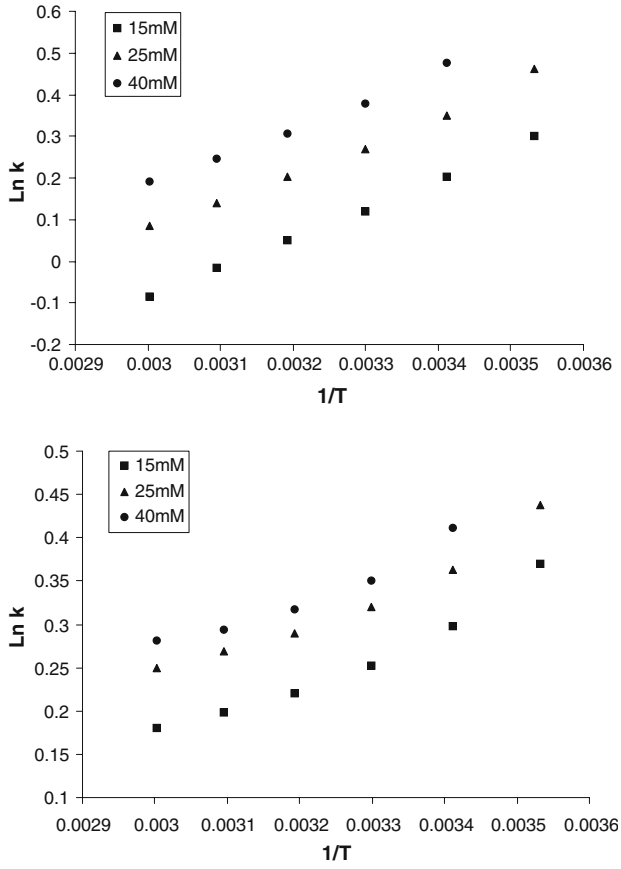

Fig. 8. Van't Hoff plots for salicyluric acid on amide phase (*top panel*) and aspartamide phase (*bottom panel*). Mobile phase: acetonitrile/water (80/20, v/v) containing 15, 25 and 40 mM ammonium acetate. Flow rate 1 mL min^{-1}, column temperature 30 °C, and UV detection at 228 nm

the ammonium acetate salt in the mobile phase. The more charged stationary phases (e.g., silica and suofobetaine phases) were more influenced by the salt concentration than the less-charged stationary phases.

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