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Influence of the mobile phase composition and pH on the chromatographic behaviour of polar neutral and ionized compounds in hydrophilic interaction chromatography

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The chromatographic behaviour of anionic, cationic and neutral analytes on the bare silica stationary phase in the hydrophilic interaction chromatography (HILIC) mode was investigated. The effect of various mobile phase parameters such as the nature and concentration of an organic solvent, pH and buffer concentration on the retention and efficiency was evaluated. The eluting strength of organic solvents follows the order: acetonitrile < isopropanol < methanol. For all analytes acetonitrile provided the highest peak efficiency. Minimal changes in the retention and efficiency of neutral compounds were observed upon changing pH (3–7) and ammonium acetate buffer concentration (0–10 mmol/L). The efficiency of charged analytes significantly increased with increasing pH and buffer concentration. The changes in the retention for cationic and anionic compounds with increasing pH and buffer concentration were in the opposite direction. The retention of anions increased, whereas an opposite trend was observed for cationic analytes.

Keywords: hydrophilic interaction chromatography, mobile phase composition, pH, retention, efficiency

INTRODUCTION

Hydrophilic interaction chromatography (HILIC) is a feasible alternative for the analysis of highly polar and ionized compounds that are poorly or even not retained in reversed-phase chromatography (RP-LC) [1–3]. This separation technique uses a polar stationary phase (for example, unmodified silica or a polar bonded phase) in conjunction with a polar mobile phase containing more than 60–70% of an organic solvent (typically acetonitrile) in an aqueous buffer. The term HILIC was first suggested by Alpert in 1990, who explained its principles and some important applications [4]. However, HILIC did not become widely recognized as a distinct chromatographic mode until it was 'rediscovered' by the scientific community in the mid of the first decade of this century [5]. The rising popularity of HILIC over the last decade coincided with a wider availability of specifically designed HILIC stationary phases with diverse functionalities, which could offer different selectivity and higher retention for polar compounds [6]. However, unmodified silica sorbents are still the most popular phases. Silica materials have also become available in sub-2 μm fully porous particles, in superficially porous particles and as monolithic columns [7].

Although HILIC has been widely applied, the retention mechanism in this separation mode is still under debate [8, 9]. The primary retention mechanism is believed to be partitioning of the analytes between a water layer

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adsorbed on the surface of the hydrophilic stationary phase and the less polar bulk mobile phase. However, secondary interactions such as hydrogen bonding, ionic and even hydrophobic interactions can also occur depending on the nature of the analyte, stationary and mobile phases [10–12]. If the sample contains a large number of analytes having different properties, then it becomes difficult to predict their chromatographic behaviour.

The aim of the current work was to investigate the effect of the mobile phase composition and pH on the chromatographic behaviour of anionic, cationic and neutral compounds under hydrophilic interaction chromatography conditions using the bare silica stationary phase.

EXPERIMENTAL

Ultra-pure water was obtained from a Mili-Q Water Purification System from Millipore (Bedford, MA, USA). Acetonitrile (ACN), methanol (MeOH), isopropanol (IPA), formic acid, acetic acid, ammonium formate and ammonium acetate were of LC-MS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetylsalicylic acid (≥99%), creatinine (≥98%), nicotine (≥99%), nicotinic acid (≥98%), acetaminophen (≥98%) and uracil (≥99%) were also from Sigma-Aldrich.

Individual stock solutions of analytes at a concentration of 200 mg/L were prepared in an ACN-water (1:1 v/v) solution. Working standard solutions at 20 mg/L were prepared prior to use by diluting the stock solution with the appropriate mobile phase. Buffers were prepared by adjusting the aqueous solution of the ammonium salt with the appropriate acid to the required pH. pH was measured in all cases before the addition of an organic solvent.

HILIC separations were performed on a Waters Acquity UPLC system (Waters, Milford MA, USA) equipped with an Acquity UPLC photodiode array detector (PDA). The Acquity UPLC BEH HILIC column (2.1 × 100 mm, 1.7 µm, Waters) was used in the experiments. The column temperature was maintained at 30°C. The mobile phase flow rate was 0.25 mL/min. The injection volume was 2 µL using a partial loop with the needle overfill injection mode. Data collection and management was performed by the Data Analysis 4.0 software (Bruker).

The column was equilibrated with each mobile phase for at least 60 min. The number of theoretical plates (*N*) was calculated using the half height method. All results were the mean of triplicate injections.

RESULTS AND DISCUSSION

Characterization of the analytes

We selected six compounds as model analytes to examine the retention and efficiency properties of the mobile phases. Their chemical structures are presented in Fig. 1. The analytes are grouped according to their charge state within the pH range 3–7 into the following three groups: cationic, anionic and neutral. Our analysis of mobile phase effects presented in this paper will focus primarily on this categorization. The pK_a and log D values of the analytes are listed in Table 1. For each group the initial separation conditions were selected and the obtained chromatograms are presented in Fig. 2. As expected, for both anionic and neutral compounds the more hydrophilic analyte with a lower log D value shows a greater retention. However, the elution order of cationic creatinine and nicotine did not match the typical HILIC elution order predicted following the increasing hydrophilicity of analytes as described by log D. This

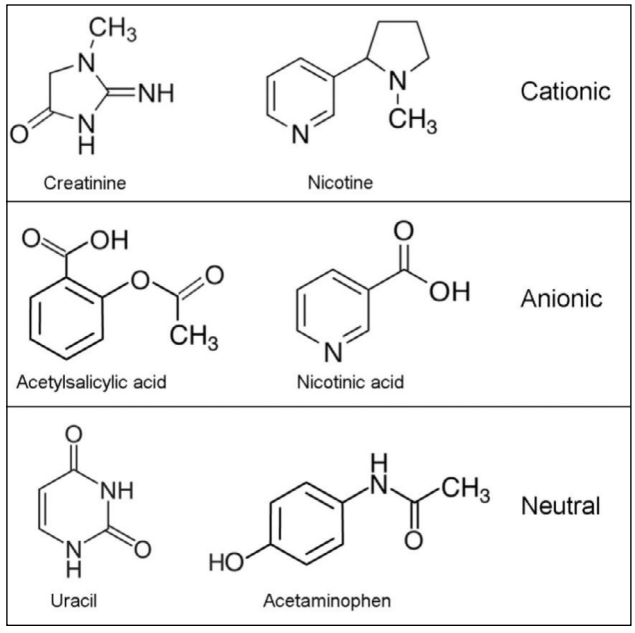

Fig. 1. Structures of the model analytes

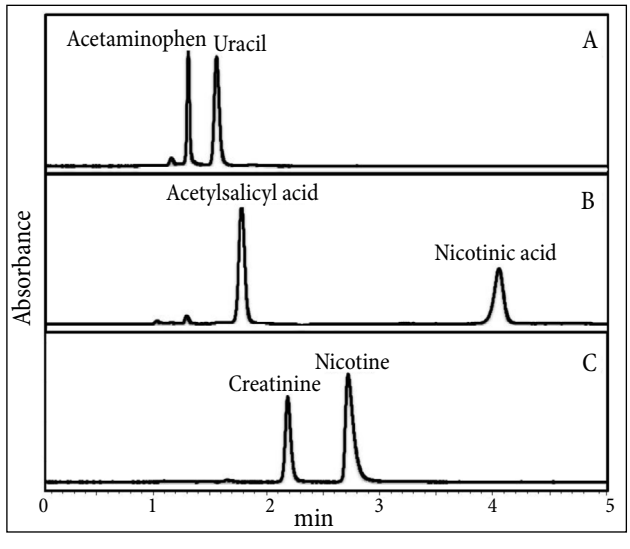

Fig. 2. Chromatograms of a pair of the model analytes obtained under initial HILIC conditions. Mobile phases: 5 mmol/L CH_3COONH_4 in 95% (A), 90% (B) and 85% (C) acetonitrile (pH 7.0). The flow rate 0.25 mL/min

behaviour was most likely due to a significant impact of secondary cation-exchange interactions between the negatively charged ionized silanols of the silica surface and the cationic analytes. However, it is difficult to deduce the exact protonation state of the analytes in the acetonitrile rich HILIC mobile phase. This complicates the prediction of the elution order for the charged compounds based on their log D values.

Table 1. **Properties of model analytes**

Analyte	pK_a	Log D
Creatinine	pK_{a1} = 4.8 pK_{a2} = 9.2	–1.02 (pH 6.8)
Nicotine	pK_{a1} = 3.1 pK_{a2} = 8.0	–0.24 (pH 6.8)
Acetylsalicylic acid	pK_a = 3.5	–0.85 (pH 6.8)
Nicotinic acid	pK_a = 2.2 (acidic)	–2.34 (pH 7.0)
Acetaminophen	9.5 (acidic)	0.25 (pH 7.0)
Uracil	9.8 (acidic)	–0.86 (pH 6.8)

The effect of an organic solvent

Mobile phases in HILIC employ oraganic-rich solvent mixtures usually containing 5–40% of water or an aqueous buffer. Typically, a minimum of about 2–3% of water is needed to enable the formation of a stagnant water layer on the surface of the polar stationary phase. In contrast to RP-LC, water is the strongest eluting solvent in HILIC but the nature of the organic solvent also has a strong influence on the separation performance. The effect of three organic solvents, namely, ACN, MeOH and IPA, on the chromatographic behaviour of the analytes was investigated. The measured retention times and the number of theoretical plates (*N*) are given in Table 2. For all analytes the eluting strength follows the order: ACN < IPA < MeOH, which is correlated with different hydrogen bonding abilities of the solvents. Polar protic solvents (MeOH and IPA) can be both a donor and an acceptor of hydrogen bonds, and aprotic ACN can be only a hydrogen bond acceptor. Consequently, protic solvents can more effectively compete for polar active sites on the surface of the stationary phase, replacing water molecules and thus producing a more hydrophobic surface. The slightly weaker elution strength of IPA compared to that of MeOH may be attributed to its less hydrophilic character (i.e. longer alkyl chain). For all analytes acetonitrile provided the highest peak efficiency. Based on the above observations, it is clearly evident that ACN is the preferred solvent for HILIC separations.

The effect of ACN concentration in the mobile phase on the retention and efficiency was investigated in the range 75–95% (v/v) at a constant ammonium acetate concentration of 5 mmol/L (pH 7). As expected, all coumpounds exhibited the typical HILIC behaviour of increasing retention with increasing the ACN content in the mobile phase (Fig. 3). As can be observed, retention times for the charged analytes are much more strongly affected by the ACN concentration than for the neutral compounds. This is possibly due to enhanced secondary analyte/stationary phase interactions (e.g. hydrogen bonding and electrostatic interactions) which may become predominant as the water content decreases [13]. Because creatinine exhibits a higher positive charge at pH 7 (see Table 1), its retention is more strongly affected by the ACN concentration than that of nicotine. As a consequence, the elution order of cationic creatinine and nicotine changes with the ACN concentration in the mobile phase. Higher ACN concentration also enchances the resolution of all analyte pairs.

Table 2. **The effect of an organic solvent on retention times and the number of theoretical plates (*N*) for test analytes**

Organic solvent	Analyte	Retention time, min	*N*
ACN	Creatinine	2.19	9460
	Nicotine	2.72	7485
	Acetylsalicylic acid	1.78	5900
	Nicotinic acid	4.05	11730
	Acetaminophen	1.31	10640
	Uracil	1.56	7450
MeOH	Creatinine	1.27	4833
	Nicotine	1.46	4918
	Acetylsalicylic acid	1.00	3642
	Nicotinic acid	1.02	3790
	Acetaminophen	1.13	5458
	Uracil	1.16	5752
IPA	Creatinine	1.52	2400
	Nicotine	1.62	1335
	Acetylsalicylic acid	1.21	738
	Nicotinic acid	2.00	1131
	Acetaminophen	1.16	1406
	Uracil	1.19	1587

The effect of mobile phase pH

The effect of the mobile phase pH on the chromatographic behaviour of model analytes was investigated in the pH range 3–7. The ammonium acetate buffer at a concentration of 5 mmol/L was utilized in the pH range 4–7. At pH < 4, the ammonium formate of the same concentration was employed due to a better buffering capacity at lower pH values. The obtained data are summarized in Fig. 4. The pH of the mobile phase determines the ionization degree of an analyte, its log D value and, consequently, its retention. In addition, the bare silica stationary phase, under certain pH conditions, also exhibits negatively charged silanol groups (pK_a ~ 4) that may interact with cationic (electrostatic attraction) and anionic (electrostatic

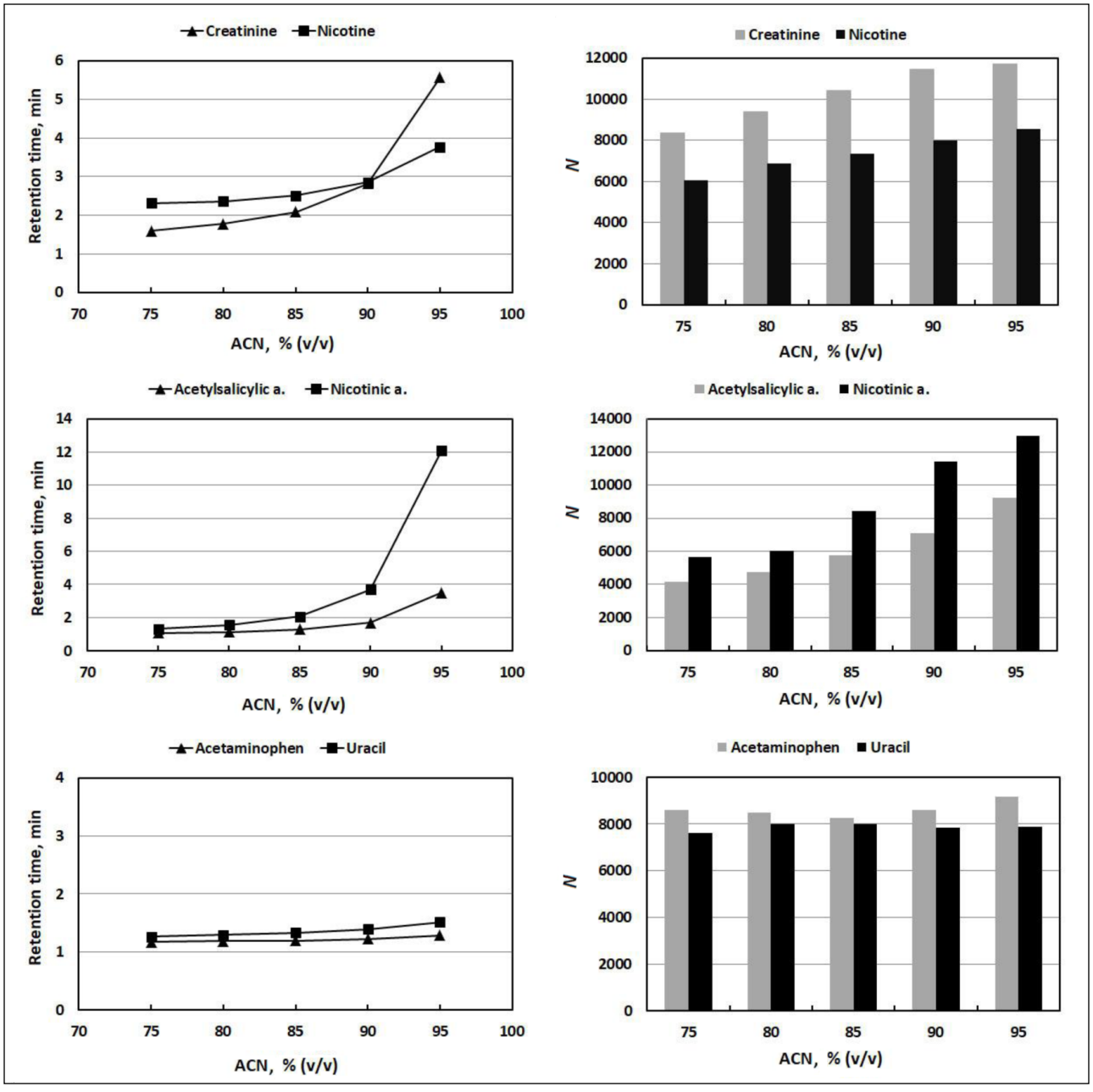

Fig. 3. The effect of ACN concentration on retention times and the number of theoretical plates (*N*) for model analytes. Mobile phase: 5 mmol/L CH_3COONH_4 in ACN/H_2O (pH 7.0)

repulsion) analytes. Thus, both these factors determine the chromatographic behaviour of a particular compound. As expected, no significant change in retention was observed for neutral analytes, which were uncharged in the working pH range. The changes in retention for cationic and anionic compounds with a change in pH, not surprisingly, are in the opposite direction, as shown in Fig. 4. When the mobile phase was less acidic, cationic bases were deprotonated and became less hydrophilic, thus leading to a decrease in retention.

The effect of pH changes on the anions are two-fold, but opposing. By deprotonating acids they become much more hydrophilic and, consequently, stronger retained. On the other hand, at higher pHs anionic analytes are also stronger repulsed from the negative charge on the silica surface and their retention is reduced. In order to be retained by hydrophilic partitioning the analyte needs to enter the stagnant water layer at the stationary phase surface. If electrostatic repulsion prevents this, retention will be low even though the acid is more hydrophilic. Thus, depending on the particular analyte/stationary phase properties, deprotonating an acid could give any outcome in terms of retention: shorter, longer or unchanged. In our system, the retention of acidic analytes increased upon increasing the mobile phase pH indicating that hydrophilic partitioning prevails on electrostatic repulsion.

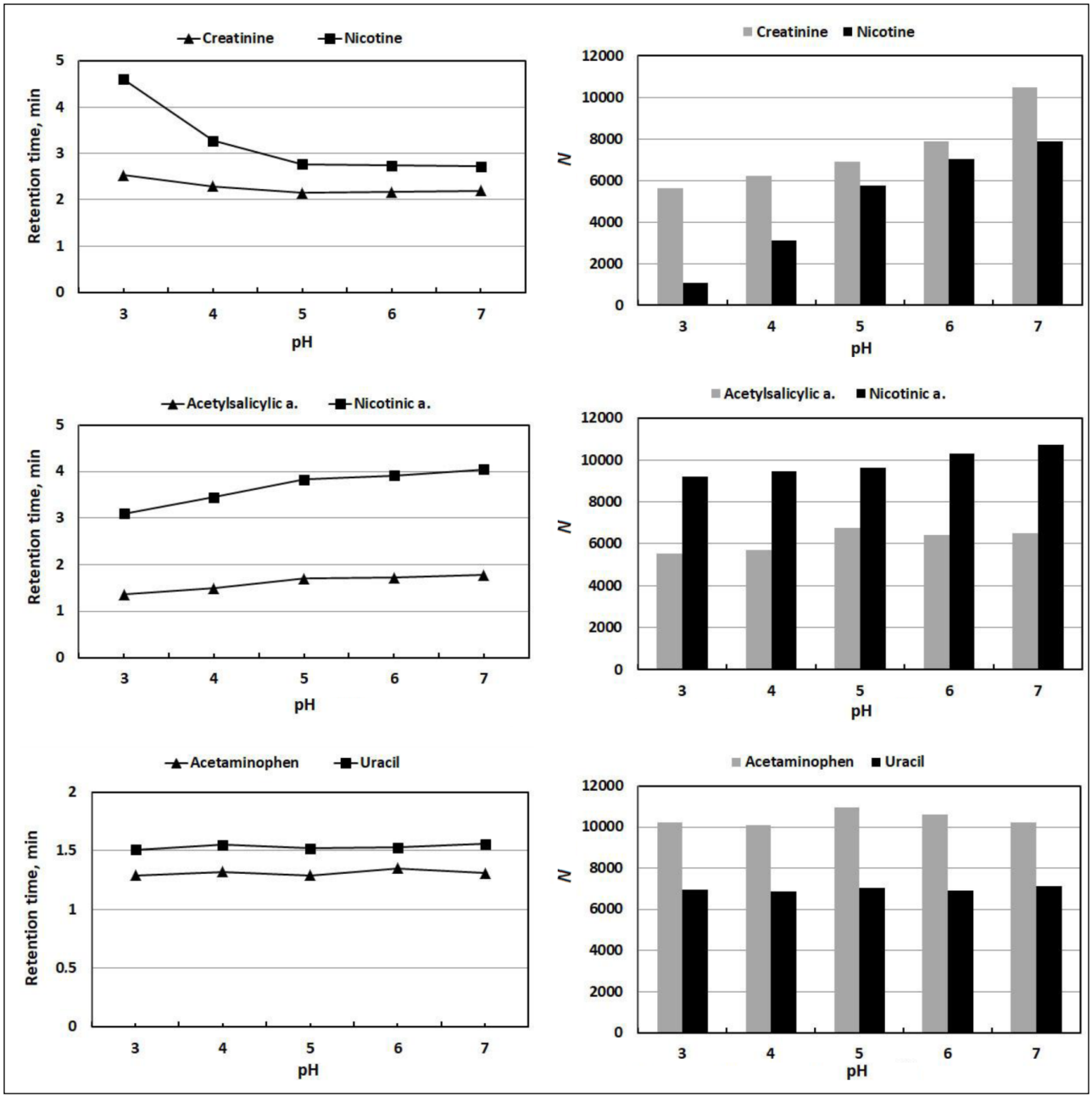

Fig. 4. The effect of mobile phase pH on retention times and the number of theoretical plates (*N*) for model analytes

In addition to retention time effects, the decrease in the mobile phase pH resulted in a significant decrease in the peak efficiency for cationic analytes. Such behaviour may most likely be attributed to the increased sample overloading effects which occur for cations more readily at lower pH values. For anionic and neutral compounds the peak efficiency fluctuated only slightly (less than 20% variation) with pH.

The effect of buffer concentration

The effect of buffer concentration on retention and efficiency was investigated by varying the concentration of ammonium acetate from 0 to 10 mmol/L, keeping the mobile phase at pH 7.0 (Fig. 5). Different trends can be observed for different analyte types. The retention of cationic compounds decreased drastically up to about 1 mmol/L, then further increases in the buffer concentration had a minor effect. In contrast, the retention of anions increased sharply in the low buffer concentrations, then more slowly in the higher concentrations of buffer. Finally, the retention of neutral analytes was practically unaffected as the buffer concentration increased: only a slight increase in retention was observed.

Increasing the buffer concentration has a general effect of decreasing the electrostatic interactions between the charged analytes and the negatively charged silica

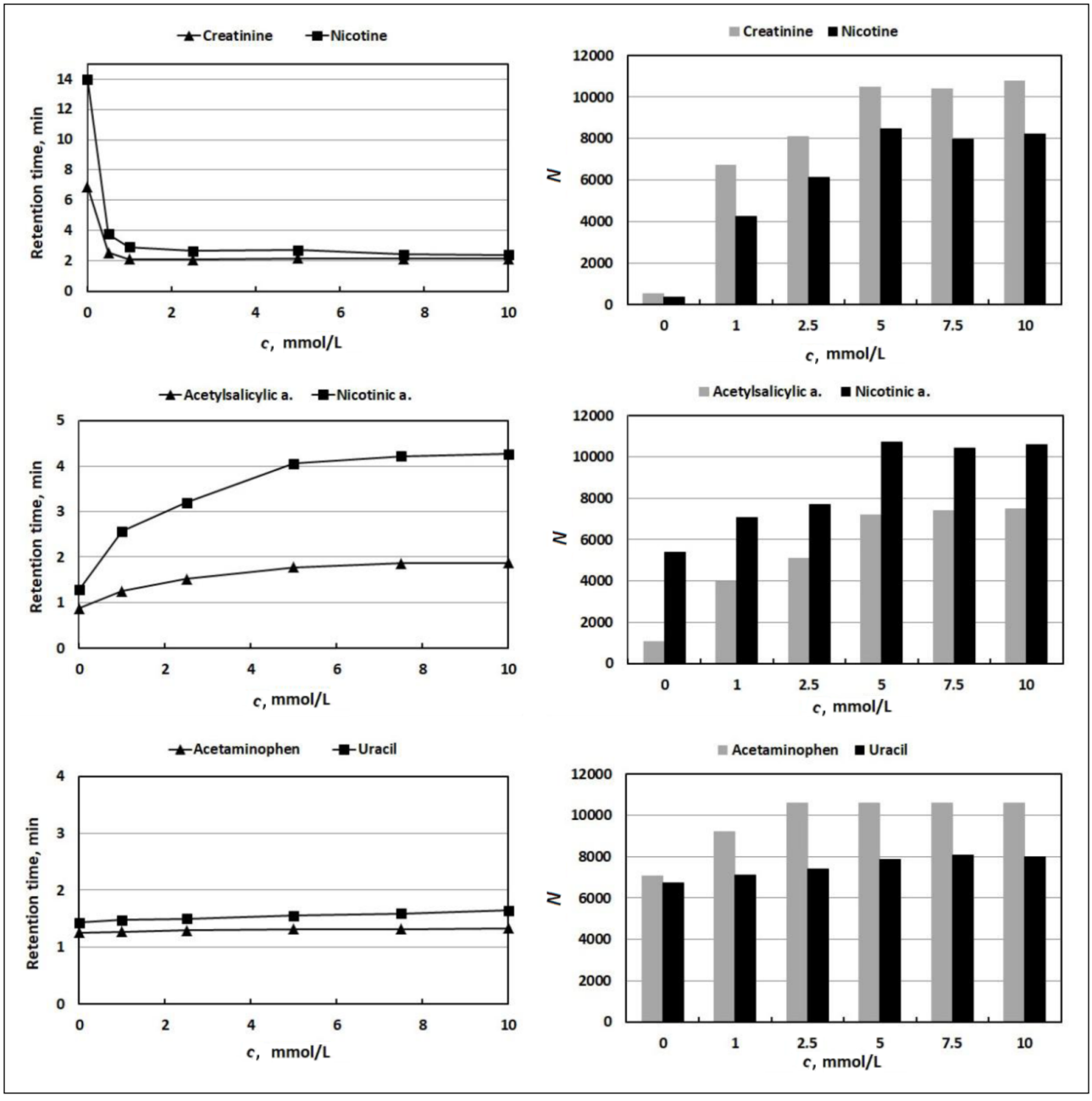

Fig. 5. The effect of buffer (CH_3COONH_4, pH 7) concentration on retention times and the number of theoretical plates (*N*) for model analytes

stationary phase surface. In the case of electrostatic attractions (cationic analytes) this leads to a decreased retention, while in the case of electrostatic repulsions (anionic analytes) the retention is increased. Neutral compounds are retained predominantly by partitioning between the water layer adsorbed on the silica surface and the less polar acetonitrile rich mobile phase. Their retention is therefore substantially less affected by the buffer concentration. A slight increase in their retention may most likely be attributed to an increase in the thickness and/or hydrophilicity of the adsorbed water layer caused by the higher concentration of solvated buffer salt ions [1]. In addition, an increased buffer concentration may also influence H-bonding and dipole–dipole interactions. Clearly, the effects of buffer concentration have only been studied at the single pH value. It is quite possible that the balance of various contributing mechanisms changes with pH, and a more comprehensive series of experiments would be necessary to examine this question further.

The peak efficiency of neutral compounds also hardly varied over the range of buffer concentrations studied. In contrast, the efficiency of charged analytes significantly increased with increasing the buffer concentration in a range of 0–5 mmol/L. For example, an increase in buffer concentration from 0 to 5 mmol/L caused between 2- (nicotinic acid) and 24-fold (nicotine) increase in efficiency. This is

presumably due to the reduction of secondary electrostatic interactions.

CONCLUSIONS

The three mobile phase parameters (concentration of organic solvent, pH and buffer concentration) enabled manipulation of the retention and efficiency of analytes with a different charge state and should be useful for the optimization of the mobile phase during HILIC method development.

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JUDRIOS FAZĖS SUDĖTIES IR pH ĮTAKA POLINIŲ NEUTRALIŲ IR JONIZUOTŲ JUNGINIŲ CHROMATOGRAFINEI ELGSENAI HIDROFILINĖS SĄVEIKOS CHROMATOGRAFIJOJE

S a n t r a u k a

Ištirta anijoninių, katijoninių ir neutralių junginių chromatografinė elgsena ant nemodifikuoto silikagelio sorbento hidrofilinės sąveikos chromatografijos sąlygomis. Įvertinta įvairių judrios fazės parametrų (organinio tirpiklio prigimties ir koncentracijos, pH, buferio koncentracijos) įtaka analičių sulaikymui ir smailių efektyvumui. Tirtų tirpiklių eliucinė geba stiprėja tokia tvarka: acetonitrilas < izopropanolis < metanolis. Visos analitės didžiausią efektyvumą pasiekia naudojant acetonitrilą. Judrios fazės pH (3–7) ir amonio acetato buferio koncentracijos (0–10 mmol/L) įtaka neutralių analičių sulaikymui ir efektyvumui nežymi. Didinant pH ir buferio koncentraciją, krūvį turinčių junginių smailių efektyvumas reikšmingai padidėja. Judrios fazės pH ir buferio koncentracijos įtaka katijonų ir anijonų sulaikymui priešinga: didinant pH ir buferio koncentraciją anijonų sulaikymas stiprėja, o katijonų – silpnėja.