Highlights

- Effect of sample solvent on peak shape in nano- and microflow HILIC was investigated.
- Sample solvent was found to increase the retention time of charged compounds.
- Compounds eluting near the solvent peak were strongly influenced by sample solvent.

A systematic investigation of the effect of sample solvent on peak shape in nano- and microflow hydrophilic interaction liquid chromatography columns

Haibin Li [a,b,c], Chusheng Liu [d], Li Zhao [a,b], Dongsheng Xu [a,b], Tingting Zhang [a,b], Qiqin Wang [a,b], Deirdre Cabooter [c*], Zhengjin Jiang [a,b*]

[a] *Institute of Pharmaceutical Analysis, College of Pharmacy, Jinan University, Guangzhou 510632, China*

[b] *International Cooperative Laboratory of Traditional Chinese Medicine Modernization and Innovative Drug Development of Chinese Ministry of Education (MOE), College of Pharmacy, Jinan University, Guangzhou 510632, China*

[c] *KU Leuven, Department for Pharmaceutical and Pharmacological Sciences, Pharmaceutical Analysis, Herestraat 49, 3000 Leuven, Belgium*

[d] *NMPA Key Laboratory for Monitoring and Evaluation of Cosmetics, Shenzhen Institute for Drug Control, Shenzhen 518057, China*

[*] Corresponding authors:

E-mail address: deirdre.cabooter@kuleuven.be (D. Cabooter), jzjjackson@hotmail.com (Z. Jiang)

Abstract

A frequently encountered problem in the practical application of nano- and microflow hydrophilic interaction chromatography (HILIC) columns is the distortion of peak shapes arising from a mismatch between the sample solvent and the mobile phase. An unmatched or improperly matched sample solvent can distort the peak shape of analytes and influence their retention times, thereby affecting the quality of the resulting chromatogram. In this work, the effect of sample solvent composition (mixtures of acetonitrile, water, methanol and isopropanol in different ratios) and injection volume (20-100 nL) was systematically investigated using a selection of neutral and charged compounds on a series of zwitterionic and charged small I.D. (0.1 mm-0.3 mm) HILIC columns. For retained compounds, pure ACN was demonstrated to be the best sample solvent to obtain narrow peaks, while for compounds that eluted very close to the solvent peak, the peak shape was distorted when the sample solvent consisted of pure ACN. A highly aqueous sample solvent, which interferes with the partitioning of polar analytes into the stationary phase, was demonstrated to be detrimental for the peak shape of retained neutral compounds, while for unretained compounds that do not or hardly interact with the stationary phase, a high amount of water in the sample solvent was not problematic. For charged compounds, water in the sample solvent favored the electrostatic attraction with the stationary phase. Therefore, the retention time of charged analytes was shown to increase with increasing water content in the sample solvent. Even when a large amount of water was present in the sample solvent, the peak shapes of these compounds were still acceptable. For highly polar compounds with a

limited solubility in aqueous sample solvents, it was found that a mixture of ACN and MeOH or IPA is a good alternative.

Keywords: Hydrophilic interaction chromatography; Zwitterionic HILIC columns; Charged HILIC columns; Peak shape; Sample solvent; Injection volume

1. Introduction

Hydrophilic interaction chromatography (HILIC), firstly proposed in 1990 [1], has become a powerful tool for the analysis of polar drugs, metabolites and biologically important compounds in proteomics, glycomics and clinical analysis [2-5]. It has gained increasing attention over the last few years due to its complementary selectivity with reversed-phase liquid chromatography (RPLC), its ability to retain polar molecules and its interesting applicability for LC-MS and 2D-LC [3, 6, 7]. The retention mechanism of HILIC is quite complex. It has been suggested that it mainly involves partitioning between the largely organic mobile phase and a liquid layer enriched with water on the surface of the hydrophilic stationary phase [8]. Moreover, other interactions can occur as well, such as ion exchange, hydrogen bonding, dipole-dipole, and even hydrophobic interactions, depending on the experimental conditions [9]. In order to better promote the properties (in terms of selectivity and peak shapes) of HILIC and improve its applicability, various chromatographic conditions and parameters can be optimized, such as the nature of the stationary phase, the mobile phase composition and temperature [9-11]. Besides that, the sample solvent is also an important factor associated with the chromatographic performance in HILIC [12]. Indeed, a mismatch between the sample solvent and the mobile phase can be very troublesome. For example, when combining RPLC and HILIC in a 2D-LC separation, portions of effluent eluting from the first dimension (^{1}D) column will be collected and transferred to the second dimension (^{2}D) column for further separation. If the ^{1}D effluent is not properly matched with the mobile phase of the ^{2}D column when the

fraction is injected, broad or distorted peaks will be observed [13-15]. It is hence important to understand the effect of the sample solvent and volume on the separation performance, when optimizing 2D-LC separations. Many studies concerning the effect of sample solvent on the peak shape in RPLC have been carried out [16-18]. In RPLC, it is suggested that the analytes should be diluted in a solvent as close as possible to, or in a weaker eluent than the mobile phase to limit band broadening as much as possible. This is particularly critical for weakly retained compounds in isocratic mode. If the sample solvent is not matched properly with the mobile phase, the peak shape can range from slightly broadened to extremely distorted or even split [16]. One possible cause for these effects is a hydrodynamic instability at the boundary of the sample solvent and the mobile phase, caused by the difference in viscosity between the two liquids [17]. Another possible reason for peak distortion is a difference in eluotropic strength between the sample solvent and the mobile phase. Peak broadening and shape abnormalities generally get worse as the sample solvent becomes stronger than the mobile phase [18]. However, these observations made for RPLC cannot simply be applied to HILIC, since the retention mechanism in HILIC is much more complex. The polar stationary phase adsorbs water from the mixed aqueous-organic mobile phase and forms a water-rich layer at its surface [1]. The perturbation of this water layer by hydrogen bonding solvents might explain why the peak shapes in HILIC separations seem to be rather sensitive to the choice of sample solvent [19]. However, only a few articles actually report sample solvent effects in HILIC as an important cause of performance loss and peak distortion in real applications. Grumbach et al. [20] used 5-

fluorouracil, uracil, 5-fluorocytosine and cytosine as model analytes, and determined that ACN:MeOH (75:25, v:v) is the best compromise to obtain a good solubility and peak shape when using 88:12 (v:v) ACN-H_2O containing 12 mM ammonium formate (pH 3) as the mobile phase. It was also demonstrated that, when it is absolutely necessary to use dimethyl sulfoxide in the sample solvent, ACN:dimethyl sulfoxide (75:25, v:v) exhibits the best results. Chauve et al. [21] used different solvent systems to dissolve acenaphthene, uracil, hypoxanthine and adenine. The best solvent system to generate satisfactory peak widths and asymmetries was ACN:water (95:5, v:v) when using ammonium formate buffer (5 mM, pH 3.0) modified with ACN (5:95, v:v) as the mobile phase. Moreover, under these analytical conditions, the separation of the model compounds dissolved in a mixture of ACN:MeOH (50:50, v:v) was better than when dissolved in ACN:water (50:50, v:v). Ruta et al. [12] investigated the effect of the sample solvent on the peak shape for small molecules (hypoxanthine, cytosine, nicotinic and procainamide) and peptides. The authors found that ACN was the best choice as sample solvent in HILIC, and water should be avoided as much as possible. It is worth mentioning that these conclusions have to be considered carefully, since the reported studies considered only a restricted number of HILIC stationary phases and analytes. Moreover, the effect of sample solvent on peak shape is more complicated when dealing with nano- and microflow HILIC columns. The geometrical volume of a nano-flow column (e.g., 0.1 mm I.D. × 150 mm length) is about 1.2 µL, while that of a standard analytical column (e.g., 4.6 mm I.D. × 150 mm length) is about 2500 µL. In order to ensure a sufficiently low injection volume, a nano volume injection valve is

116 required. To the best of our knowledge, the smallest internal loop for sample injectors
117 available on the market is 4 nL, while a 20 nL internal loop is more generally used for
118 nano HPLC [22-24], which is about 1.7% of the geometrical column volume of a 0.1
119 mm I.D. × 150 mm column. This corresponds with an injection volume of roughly 42
120 μL on a standard analytical column (4.6 mm I.D. × 150 mm length). Clearly, this
121 relatively large volume of sample solvent may lead to peak distortions. Therefore, it is
122 of great interest to study the effects of sample solvent in nano- and microflow HILIC
123 using different types of columns and analytes for a better understanding of the
124 phenomenon and for practical applications.
125 In this study, a variety of commercial and lab-made HILIC columns with inner
126 diameters of 0.1 and 0.3 mm were investigated to obtain a better understanding of the
127 relationship between the choice of sample solvent and the resulting peak shapes of both
128 neutral and charged analytes. The effect of various polar protic solvents (MeOH and
129 IPA) was also investigated in terms of resulting peak shapes.

130 **2. Materials and methods**

131 **2.1 Reagents and materials**

132 Toluene, thymine, uracil, 5-methyluridine, uridine, cytosine, thiourea, propranolol
133 hydrochloride, oxprenolol hydrochloride, ammonium formate, formic acid and
134 ammonium hydroxide were obtained from Sigma-Aldrich (Shanghai, China). HPLC-
135 grade ACN, MeOH and IPA were purchased from CNW (Shanghai, China). Distilled
136 water was deionized using a Millipore Milli-Q system (Bedford, MA, USA).
137 The columns employed in this study were a ZIC-HILIC column (0.3 mm I.D. × 150

mm, 3.5 μm, Merck), a ZIC-cHILIC column (0.3 mm I.D. × 150 mm, 3 μm, Merck), and several lab-made zwitterionic monolithic capillary columns previously reported by our group, namely poly(SPDA-co-MBA) [25], poly(SPE-co-MBA), poly(MMPC) [26], poly(SPP-co-MBA) [27] monolithic columns, and a sulfopropyl poly(SPA-co-MBA) monolithic column, and a quaternary ammonium poly(AETA-co-MBA) monolithic column [28]. The dimensions of all these capillary columns were 0.1 mm I.D. × 150 mm.

2.2 Instrumentation

All nano- or micro-HPLC experiments were performed in isocratic mode, carried out on a self-assembled nano-HPLC system, composed of an Applied Biosystems Spectroflow 757 UV detector (California, USA) with a lab-made on-column detection cell, a DiNa-S nano pump (flow range: 1 to 100 000 nL min^{-1}) (KYA Technologies, Tokyo, Japan), a Spark HOLLAND autosampler (Emmen, Netherland) equipped with either a six-port injection valve with a 1 μL external loop or a four-port injection valve with a 20 nL internal loop (both from Valco, Houston, USA). Data acquisition and data handling were performed using a HW-2000 chromatography workstation (Qianpu Software, Shanghai, China). The pH values were measured using a Sartorius PB-10 pH meter (Göttingen, Germany). All chromatograms were transferred into .txt files and then redrawn using Microcal Origin 9.0.

2.3 Sample preparation

Stock solutions (1 mg mL^{-1}) of each analyte were prepared by dissolving the corresponding compounds in ACN:H_2O (50:50, v:v). All final dilutions were performed

with the appropriate sample diluent, to have the largest possible proportion of the desired sample solvent. Thus, the quantity of solvent used for the preparation of the stock solution was negligible. All solutions were filtered through a 0.45-μm membrane prior to experiments.

2.4 Chromatographic conditions

Stock solutions of ammonium formate (500 mM) were prepared and adjusted to the desired pH value with 1 M ammonium hydroxide or 1 M formic acid. Unless otherwise noted, the mobile phase pH values mentioned throughout this research refer to the aqueous portion only. Mobile phases were prepared by pre-mixing the desired amount of ACN, water and ammonium formate solution. All mobile phases were filtered through a 0.45-μm membrane prior to experiments.

Toluene was used as the dead time marker. Retention factors (k) of solutes were calculated as follows:

$$k = \frac{t_R - t_0}{t_0}$$

where t_R (min) is the retention time of the solute and t_0 is the dead time (min). The flow rate was constant throughout the experiments. For the 0.1 mm I.D. columns, the flow rate was 800 nL min^{-1}, while 4000 nL min^{-1} was used for the 0.3 mm I.D. columns. Unless otherwise noted, the injection volume was 20 nL. Each sample was injected three times consecutively. The RSD obtained on the retention times was never higher than 2.45%.

3. Results and discussion

3.1 Analysis of neutral compounds

Except for partitioning, other retention mechanisms such as ion exchange, hydrogen bonding, dipole-dipole and even hydrophobic interactions can occur in HILIC, depending on the experimental conditions. The retention mechanism depends on the type of stationary and mobile phase, as well as the character of the analyte (e.g., polarity and ionization rate). In order to systematically investigate the influence of various factors, neutral compounds were investigated first.

3.1.1 Neutral compounds eluting after the solvent peak

Uracil, 5-methyluridine, uridine, cytosine and thiourea were selected as model compounds. As can be deduced from Table 1, their pKa values are between 8.95 and 14.91. Under the HILIC mobile phase conditions employed here (ACN:H_2O, 93:7, v:v), all five analytes were neutral and their retention factors ranged between 0.44 to 3.68. To better understand the effect of the sample solvent on the peak shape of these neutral compounds, the five model analytes were dissolved in different mixtures of ACN:H_2O (100:0, 90:10, 80:20, 70:30 and 60:40, v:v), respectively. Toluene, pure water and pure ACN were injected individually to identify the column dead time and the solvent peaks of water and ACN. Fig. S1 in the Supporting Information shows an example of the obtained peaks on the poly(SPDA-co-MBA) monolithic column. Compared with the elution time of toluene (t= 2.20 min), and ACN (t= 2.26 min), a slightly stronger elution time was observed for water (t= 2.39 min), which is most likely due to its interaction with the polar stationary phase.

As shown in Fig. 1, the best peak shapes for uracil, 5-methyluridine, uridine, cytosine and thiourea were obtained when they were dissolved in pure ACN. A similar

observation has been reported by Ruta et al. [12]. When the solubility of the polar compounds is not limited in ACN, pure ACN should therefore be the first choice of sample solvent for neutral compounds in HILIC. An increasing amount of water in the sample solvent clearly has a negative effect on the peak shapes of the five analytes. When the water content is too high (≥ 20% for $k \leq 0.82$ and ≥ 30% for $0.82 < k \leq 1.60$), the peaks start to split. The obtained peak profiles for uracil, 5-methyluridine, uridine and cytosine when using ACN:H_2O (60:40, v:v) as the sample solvent, seem to depend largely on their retention factor (Fig. 1A-D). Uracil, with a retention factor of k= 0.44, eluted very close to the water peak. The front end of the uracil peak was higher than the rear end, while the front end was relatively lower for the more retained compounds (i.e., 5-methyluridine and uridine). No peak splitting was observed for the most retained compound (i.e., cytosine), although the peak was severely broadened. The impairment in peak shape was more obvious for compounds with a low retention factor, which corresponds with the observation in RPLC where compounds eluting with large k values are less influenced by the early-eluting solvent band [18]. Except for the distorted peak shape, highly aqueous sample solvents also resulted in a generally reduced retention. The front end of the peak eluted faster while the rear end had the same retention time as the compound when using pure ACN as sample solvent (Fig. 1A, 1B and 1C).

The mechanisms explaining the above observations are visualized in Fig. S2 in the Supporting Information. When the sample is dissolved in pure ACN, the analyte will enter the column together with the sample solvent (pure ACN), which at this stage can

226 be regarded as part of the mobile phase. Since the eluotropic strength of pure ACN is
227 weak, any compound within the sample solvent that is even mildly hydrophilic (and
228 hence attracted more strongly to the stationary phase) will be “trapped” on the
229 stationary phase at the head of the column. The eluotropic strength of the eluent system
230 will increase as the mobile phase enters the column and the analyte band will begin to
231 migrate through the stationary phase. The migration velocity of the analyte band should
232 be lower than that of the ACN band, since the analyte band is moved by the mobile
233 phase and retained by the stationary phase. For poorly retained compounds, the front of
234 the analyte band (nearest the UV detector) will be dissolved in ACN while the rear of
235 the analyte band (nearest the sample injector) will be dissolved in mobile phase. The
236 eluotropic strength at the front of the analyte band will always be slightly weaker than
237 that at the rear of the analyte band. This causes a further “focusing” process whereby
238 the analyte band is compressed as the rear part of the band moves more quickly than
239 the front (Fig. S2A). When ACN is replaced with a highly aqueous sample solvent, the
240 highly aqueous sample solvent will impair analyte partitioning into the stationary phase,
241 at least partially, and will carry part of the sample through the column at a higher elution
242 speed due to the higher eluotropic strength at the front of the peak, leading to a decrease
243 in elution time for (that part of) the sample. At the same time, that part of the sample
244 that can diffuse out of the solvent band and into the mobile phase, will be retained on
245 the stationary phase and elute with the same elution time as observed for the sample
246 dissolved in pure ACN (Fig. S2B). The higher the amount of water in the sample solvent,
247 and the lower the retention factor of the compound, the more pronounced this effect is.

For the least retained compound (uracil), the majority of the analyte molecules will remain within the solvent band leading to the higher observed front end of the peak. Interestingly, the negative effect of the highly aqueous sample solvent on the peak shapes of the least retained compounds (uracil and 5-methyluridine, Fig. 1A-B) was more pronounced than for thiourea that had a similar retention factor (Fig. 1E). This is presumably due to the fact that the solubility of thiourea in water (see Table 1) and ACN is significantly higher than for uracil and 5-methyluridine, allowing thiourea to diffuse more readily out of the sample solvent and into the mobile phase. It has also been demonstrated that the diffusion coefficient of thiourea is some 1.2-1.3 times higher than that of uracil for ACN:H_2O mixtures ranging in composition between 100:0 and 0:100 (v:v) [29]. This is presumably why thiourea does not demonstrate a split peak to the same extend as uracil and 5-methyluridine. To sum up, the above examples demonstrate that the effects of highly aqueous injection solvents are different for different compounds and depend both on their retention factor and physical properties.

3.1.2 Neutral compounds eluting near the solvent peak

Less polar compounds interact less with the stationary phase, and therefore have a smaller retention factor in HILIC. After injection, these compounds will have a similar linear velocity as the sample solvent as most molecules of these compounds will stay within the sample solvent plug throughout the column. Therefore, it is expected that the sample solvent will have a large impact on compounds eluting near the solvent peak. Thymine is a weakly retained solute in HILIC, with a log P value of -0.621. Fig. 2 shows the elution of thymine on four different columns. The retention factors of

thymine on the four HILIC columns differed between k= 0.27 and k= 0.56. The peak profile of thymine on the poly(MMPC) monolithic column (k= 0.37) and the poly(SPA-co-MBA) monolithic column (k= 0.56) (Fig. 2C & 2D), was similar to the peak profile of uracil on the poly(SPDA-co-MBA) monolithic column (k= 0.44) (Fig. 1A), because their retention factors were similar. In all cases, pure ACN was the best sample solvent to obtain good peak shapes. However, a different phenomenon was observed on the poly(SPDA-co-MBA) and poly(SPE-co-MBA) monolithic columns (Fig. 2A & 2B). The peak shape was not the best and the retention time increased when pure ACN was used as the sample solvent. Fig. 3 showed the chromatogram of a mixture of thymine, uracil, 5-methyluridine and uridine on the poly(AETA-co-MBA) monolithic column. The effect of the sample solvent on thymine was obviously special comparing with other three compounds. A fronting and narrow peak was observed when using pure ACN as the sample solvent and the retention time was significantly delayed in comparison with sample solvents containing water. The mechanism explaining the above observation is visualized in Fig. S3 in the Supporting Information. When the sample solvent is pure ACN, there will be some interaction between the analyte and the stationary phase, trapping the analyte on the stationary phase at the head of the column and hence slightly delaying its elution. Moreover, the front of the analyte band will be dissolved in ACN, while the rear of the analyte band will be dissolved in mobile phase. Since the eluotropic strength at the front of the analyte band will be slightly weaker than that at the rear of the analyte band, the migration velocity at the rear end of the band will be faster, resulting in compression of the analyte band. It is recommended to

use the mobile phase as the sample solvent instead of pure ACN for the compound eluting near the solvent peak.

When the percentage of water in the sample solvent increases, the peak width of thymine shows a different behavior, depending on the retention factor. The peak remains narrow, when is coeluted with the solvent band, or broadens for slightly higher retention factors, or compresses, when a part of the analyte band migrates before the solvent band. These phenomena can be explained by the different migration velocities between the analyte band and the sample solvent under different conditions (e.g., different mobile phases or stationary phases). When the analyte is coeluted with the solvent band, the analyte will remain within the sample solvent. In this case, the physical behavior of the rear end and front end of the peak should be the same, and the peak profile will be normal (Fig. 2A & 2B). When the analyte is slightly retained, the analyte will (partly) interact with the stationary phase and (part of) the analyte will hence move slower than the sample solvent, leading to distorted peak profiles (Fig. 2C & 2D) as explained in section 3.1.1. When a part of the analyte band migrates before the solvent band and the rear part overlaps with the solvent band, the eluotropic strength at the rear of the analyte band will be slightly stronger than that at the front of the analyte band, resulting in compression of the analyte band (Fig. 3).

3.2 Analysis of charged compounds

Charged compounds are an important kind of polar compounds. Positively or negatively charged molecules are hydrophilic because they are attracted to partially charged water molecules. The retention mechanism of charged compounds is complex.

Ion exchange may occur between the analytes and the stationary phase. To investigate this further, four HILIC columns derivatized with different functional groups (zwitterionic and sulfopropyl) were selected.

3.2.1 Charged compounds eluting near or just before the solvent peak

Two β-blockers (propranolol and oxprenolol) were used as test compounds. Their pKa values are about 9.5 (Table 1). The effect of the sample solvent was first studied using a zwitterionic HILIC column (poly(SPP-co-MBA)). The zwitterionic HILIC column has both negatively and positively charged groups and has a very weak surface charge. ACN:H_2O (90:10, v:v) and ACN:20 mM ammonium formate (pH 4.0) (90:10, v:v) were used as the mobile phases, respectively. Propranolol and oxprenolol are partially ionized and positively charged in ACN:H_2O (90:10, v:v) while fully ionized at pH 4.0. Despite this, the retention time of the two compounds was very low in both mobile phases (Fig. 4). Propranolol and oxprenolol eluted very early (near or just in front of the water peak). The retention time of the analytes increased slightly when the percentage of water in the sample solvent was increased. However, the peak profiles were not affected by the sample solvent. As suggested by their retention times, propranolol and oxprenolol did not interact with the stationary phase and stayed in the mobile phase at all times. The migration velocity of the analyte band and the sample solvent band was the same, so the peak profiles were not affected by the sample solvent.

3.2.2 Charged compounds eluting near and after the solvent peak

In order to increase the electrostatic interaction between the analytes and the columns, a negatively charged hydrophilic column (poly(SPA-co-MBA)) was used next. In

ACN:H_2O (90:10, v:v), the analytes were partially ionized and the electrostatic attraction was relatively weak, so the retention times of the analytes were very small and eluted near the solvent peak (Fig. 5A & 5B).

When using pure ACN as the sample solvent, the retention time was slightly delayed due to a slight trapping of the analytes on the stationary phase, and the peak width was narrow, similar to what was observed in Fig. 2A & 2B. When the percentage of water in the sample solvent increased, there was no longer any interaction with the stationary phase and the analyte band stayed within the sample solvent throughout its passage through the column, leading to a normal peak profile. When the mobile phase was changed to ACN:20 mM ammonium formate (pH 4.0) (90:10, v:v), propranolol and oxprenolol were fully ionized and a strong electrostatic attraction occurred between the positively charged compounds and the negatively charged stationary phase. The salt moreover increased the hydrophilic partitioning into the stationary phase by increasing the volume of the water layer [30], increasing the retention of the analytes significantly (Fig. 5C & 5D). The peak profiles were good when using pure ACN as the sample solvent and no significant delay of the retention time was observed. Since the migration velocities of the analyte band and ACN were very different under these conditions, the analyte band was able to leave the ACN sample band quickly. Adding 10% of water to the sample solvent led to a slight distortion of the peak shape. However, when further increasing the concentration of water in the sample solvent, the retention time of the peak increased. This phenomenon was not observed for the neutral compounds. The analyte retention mechanism is not reversed from hydrophilic to hydrophobic

358 interaction on the poly(SPA-co-MBA) column when the amount of water in the mobile
359 phase increases, as has been demonstrated in a previous study [28]. So, it is believed
360 that the increased elution time is caused by electrostatic attraction. To further
361 investigate this, the pH of the mobile phase was changed to 7.0 and the water in the
362 sample solvent was increased to maximum 100% (Fig. S4). Since the analytes are
363 partially ionized at pH 7.0, the electrostatic attraction should be weaker, as evidenced
364 by the elution times of propranolol in Fig. 5C and Fig. S4 when using for example
365 ACN:H_2O=60:40 as the sample solvent. This supports the above hypothesis that the
366 decreasing migration velocity was caused by electrostatic attraction.
367 To investigate why the sample solvent changes the strength of the electrostatic
368 attraction, the retention of propranolol in several mobile phases containing different
369 ratios of ACN and 20 mM ammonium formate (pH 7.0) was investigated (Fig. 6). The
370 retention time of propranolol decreased as the percentage of the buffer was increased
371 from 5% to 20% and then increased again with further increment of buffer. A similar
372 phenomenon has also been observed when analyzing negatively charged compounds
373 on an amino column [30]. The electrostatic interaction between the solutes and the
374 stationary phase becomes less important at highly organic mobile phases, where
375 hydrophilic partitioning will dominate the retention mechanism. When the organic
376 content in the mobile phase decreases, hydrophilic partitioning becomes less important
377 and electrostatic attraction will dominate the retention mechanism. This could be
378 related to a competition between the positively charged compounds and the ammonium
379 cations in the mobile phase for the cation-exchange sites of the column. At highly

organic mobile phases, the positively charged compounds and the ammonium cations partition more efficiently into the water-rich layer and hence may be rendered more competitive for the negatively charged sites of the stationary phase. The decreasing migration velocity of the peaks of propranolol and oxprenolol indicates that when highly aqueous sample solvents move through the column, the mobile phase eluting close the solvent band will mix with the sample solvent and its water content will increase (Fig. S5).

The effect of the sample solvent was also investigated on the other two zwitterionic silica columns (ZIC-HILIC & ZIC-cHILIC). An increased retention of propranolol was observed on the ZIC-HILIC column when the water content in the sample solvent was increased. A peak appeared near the position of the solvent peak. The height of this peak was much higher than that of the water peak (Fig. S6A). This indicates that the peak is propranolol rather than the solvent peak. Other authors have observed similar unretained peaks and referred to them as "breakthrough peaks" [31-33]. The reason for the breakthrough peak is the strong elution capacity of the injection solvents. If the retention factor is sufficiently low and when the injection volume is large enough, some of the sample molecules will remain in the injection solvent band while passing through the column, and will be detected as a "breakthrough" peak [32]. The injection volume (0.2 μL) was relatively large for the ZIC-HILIC column, therefore, a breakthrough peak was observed in this column. A conceptual drawing of the migration process of the solvent band and the analyte band in the ZIC-HILIC is shown in Fig. S7. The solvent band can be divided into three regions due to the diffusion process of the sample solvent,

a front part, a central part and a rear part. The water content in the central part was very high and the eluotropic strength was low because of the increased electrostatic attraction between the analytes and the stationary phase. The water content in the front part and rear part were low, therefore the eluotropic strength of these two parts was high as the partitioning of the analytes into the stationary phase was affected as explained before. The water content in the front part of the solvent band was always low, so a part of the analyte band migrated with the solvent at all time and was eventually observed as the breakthrough peak.

The ZIC-cHILIC column contains phosphorylcholine functional groups, and a positive quaternary ammonium group is positioned at the end of the functional molecule. The retention of the positively charged propranolol was hence weaker on this column due to electrostatic repulsion. For this reason, no increased retention of propranolol was observed on the ZIC-cHILIC column (Fig. S6B). The reason why the peak areas were not the same is discussed in section 3.4.

3.3 Effect of alternative organic solvents

As demonstrated above, the weak organic solvent ACN generally is a good choice as sample solvent in HILIC analyses. However, analyte solubility in ACN is often a major issue in HILIC. Thus, the effect of various polar protic solvents (MeOH and IPA) was investigated in terms of resulting peak shapes. Mixtures of ACN and MeOH (or IPA) in different proportions were used as the sample solvent. Thiourea, uracil, and thymine were selected as model compounds. The study was first performed on the poly(SPE-co-MBA) monolithic column.

For thiourea (k= 1.38) (Fig. 7) and uracil (k= 0.45) (Fig. 8), ACN was a good sample solvent to obtain a good peak shape. When water was added to the sample solvent, the peak profile was broadened (thiourea) or distorted (uracil). However, the peak shape was very good when using a mixture of ACN and MeOH or IPA as sample solvents. No obvious decrease of chromatographic performance could be observed. Water has the strongest eluotropic strength in HILIC while MeOH and IPA are relatively weaker. The peaks of uracil and thiourea did not overlap with the peaks of MeOH and IPA, which means that the analyte band left the solvent band during the migration in the column. For these reasons, the effect of MeOH and IPA on the partitioning of the analytes into the stationary phase was limited. A mixture of ACN and MeOH or IPA is hence a good way to limit the use of water in the sample solvent of retained compounds. Other studies have made a similar point [12, 34].

However, for the weakly retained compound thymine (k= 0.27) (Fig. 9), mixtures of ACN and MeOH or IPA as the sample solvent were not a good choice to get a good peak shape. The peak was distorted and an uninterrupted, double-headed peak was observed with increasing MeOH or IPA content in the sample solvent. The retention times of thymine and the MeOH (or IPA) peak were very close. A part of the analyte band hence overlapped with the solvent band. The components of the sample solvent were different from the mobile phase. It is believed that when MeOH (or IPA) enter the column from the sample loop and mix with the mobile phase, the interface of the solvent band is very complex (as indicated by the baseline fluctuation between 1.5-2.8 min indicating a positive and negative peak).

The effect of alternative organic solvents was also investigated on another column (poly(SPP-co-MBA) monolithic column). Similar phenomena as discussed above were observed. Mixtures of ACN and MeOH or IPA were good choices as samples solvents to get good peak shapes (Fig. S8 & S9) except for weakly retained compounds (Fig. S10).

3.4 Effect of the injection volume

A large injection volume can be beneficial to obtain a higher detection signal for lowly concentrated samples, as well as to increase the sample load in preparative chromatography. The sample solvent will mix with the mobile phase after injection. The larger the injection volume, the more time it will take for mixing, and the more obvious the effect of the sample solvent on the peak shape will be. Fig. 10 compares the sample solvent effect for different injection volumes (20 nL and 100 nL). The dimensions of this column are 0.3 mm I.D. × 150 mm. When the injection volume was 20 nL (0.2% of the geometrical column volume), the effect of the solvent on the peak shape was limited. However, when the injection volume was increased to 100 nL (1% of the geometrical column volume), the increasing water content in the sample solvent resulted in peak broadening. A hydrodynamic instability may occur at the boundary of the sample solvent and the mobile phase, if the viscosities of the sample solvent and the mobile phase vary a lot. As shown in Fig. S6 & Fig. 10B, when the water content of the sample solvent was high, the peak area of the analyte was very large. Presumably because the mode of injection was Partial Loop-fill Injection, and the hydrodynamic

467 instability let more sample enter the sample loop. The difference in viscosity between
468 the two liquids is a common challenge in HILIC during the injection.

469 **4. Conclusion**

470 The effect of the sample solvent on the peak shape in nano- and microflow HILIC
471 columns was investigated systematically. The relatively large injection volume makes
472 the effect of the sample solvent more important in nano- and microflow LC, compared
473 to narrow and standard bore columns. Neutral and charged compounds were used as
474 test compounds.

475 If the solubility of a compound in ACN is not an issue and the retention time is not close
476 to that of the solvent peak, ACN is the best sample solvent to obtain narrow peaks.
477 Using highly aqueous sample solvents leads to poor peak shapes for retained
478 compounds, as it impairs analyte partitioning into the stationary phase. This can result
479 in peak broadening, peak splitting, loss of resolution, reduced retention, or even
480 increased retention. For unretained compounds, that do not or hardly interact with the
481 stationary phase, a high amount of water in the sample solvent is not problematic. A
482 high amount of water in the sample solvent also seems to increase the electrostatic
483 interaction between charged compounds and a charged stationary phase.

484 A mixture of ACN and MeOH or IPA is a good way to limit the use of water in the
485 dilution solvent for retained compounds that do not dissolve well in pure ACN. Another
486 option is to decrease the injection volume.

487 The selection of the sample solvent is much more important for compounds with a low
488 retention factor. If the compounds have a similar migration velocity as the solvent, the

peak shape may be distorted when using ACN as the sample solvent. In this case, it is recommended to use the mobile phase as the sample solvent.

Acknowledgements

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632 **Figure captions**

633 **Figure 1.** Effect of sample solvent composition on compounds eluting after the solvent
634 peak. Experimental conditions: column: poly(SPDA-co-MBA) monolithic column;
635 mobile phase: ACN:H_2O (93:7, v:v); sample: A) uracil (k= 0.44), B) 5-methyluridine
636 (k= 0.82), C) uridine (k= 1.60), D) cytosine (k= 3.68), E) thiourea (k= 0.65); detection
637 wavelength: 254 nm (except for water: 190 nm).

638

639 **Figure 2.** Effect of sample solvent composition on a compound eluting near the solvent
640 peak. Experimental conditions: mobile phase: ACN:H_2O (93:7, v:v); sample: thymine;
641 detection wavelength: 254 nm (except for water: 190 nm); columns: A) poly(SPDA-co-
642 MBA) monolithic column (k= 0.27), B) poly(SPE-co-MBA) monolithic column (k=
643 0.27), C) poly(MMPC) monolithic column (k = 0.37), D) poly(SPA-co-MBA)
644 monolithic column (k= 0.56).

645

646 **Figure 3.** Comparison of sample solvent effect on compounds eluting near and after
647 the solvent peak. Experimental conditions: mobile phase: ACN:H_2O (93:7, v:v); sample:
648 1) thymine, 2) uracil, 3) 5-methyluridine, 4) uridine; detection wavelength: 254 nm;
649 columns: poly(AETA-co-MBA) monolithic column.

650

651 **Figure 4.** Effect of sample solvent composition on unretained charged compounds.
652 Experimental conditions: column: poly(SPP-co-MBA) monolithic column; detection
653 wavelength: 230 nm; A) mobile phase: ACN:H_2O (90:10, v:v), sample: propranolol, B)

mobile phase: ACN:H_2O (90:10, v:v), sample: oxprenolol, C) mobile phase: ACN:20 mM ammonium formate (pH 4.0) (90:10, v:v), sample: propranolol, D) mobile phase: ACN:20 mM ammonium formate (pH 4.0) (90:10, v:v), sample: oxprenolol.

Figure 5. Effect of sample solvent composition on charged compounds eluting near and after the solvent peak. Experimental conditions: column: poly(SPA-co-MBA) monolithic column; detection wavelength: 230 nm; A) mobile phase: ACN:H_2O (90:10, v:v), sample: propranolol, B) mobile phase: ACN:H_2O (90:10, v:v), sample: oxprenolol, C) mobile phase: ACN:20 mM ammonium formate (pH 4.0) (90:10, v:v), sample: propranolol, D) mobile phase: ACN:20 mM ammonium formate (pH 4.0) (90:10, v:v), sample: oxprenolol.

Figure 6. The retention of propranolol in several mobile phases containing different ratios of ACN and 20 mM ammonium formate (pH 7.0). Experimental conditions: column: poly(SPA-co-MBA) monolithic column; detection wavelength: 230 nm; sample: propranolol, sample solvent: ACN.

Figure 7. Effect of MeOH and IPA in the sample solvent on the resulting peak shape of thiourea (k=1.38). Experimental conditions: column: poly(SPE-co-MBA) monolithic column; detection wavelength: 254 nm; mobile phase: ACN:H_2O (93:7, v:v).

Figure 8. Effect of MeOH and IPA in the sample solvent on the resulting peak shape of

uracil (k= 0.45). Experimental conditions: column: poly(SPE-co-MBA) monolithic column; detection wavelength: 254 nm; mobile phase: ACN:H_2O (93:7, v:v).

Figure 9. Effect of MeOH and IPA in the sample solvent on the resulting peak shape of thymine (k= 0.27). Experimental conditions: column: poly(SPE-co-MBA) monolithic column; detection wavelength: 254 nm; mobile phase: ACN:H_2O (93:7, v:v).

Figure 10. Comparison of the extent of the effect of the sample solvent composition on the resulting peak shape when using different injection volumes. Experimental conditions: column: ZIC-cHILIC; detection wavelength: 254 nm; mobile phase: ACN:H_2O (90:10, v:v); sample: 5-Methyluridine (k= 0.82); injection volume: A) 20 nL, B) 100 nL.

Table 1. Physicochemical properties of the analytes. These properties were taken from the database of SciFinder (www.scifinder.cas.org)

Figure 1

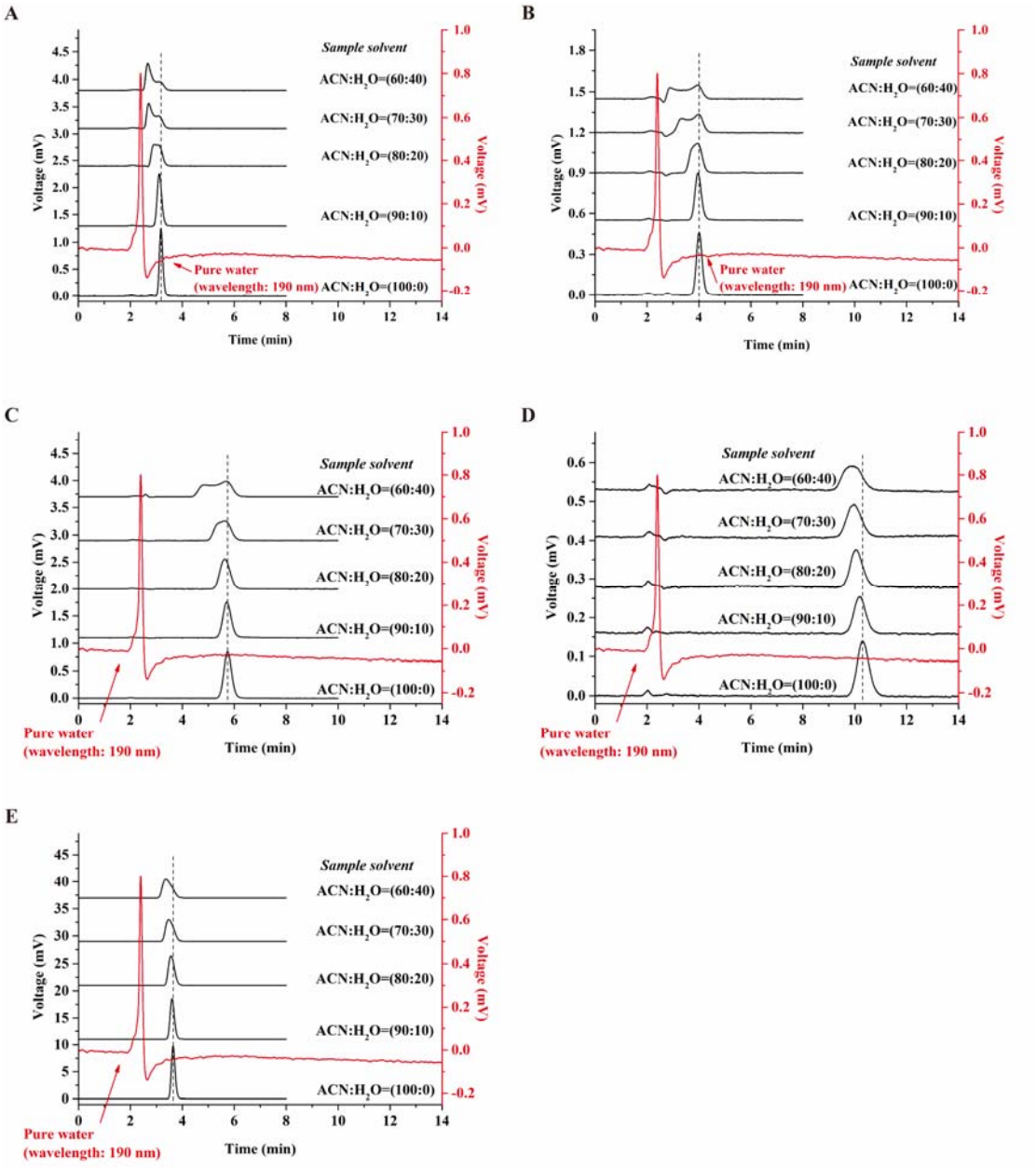

A
B
C
D
E
Sample solvent
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Pure water
(wavelength: 190 nm)
Voltage (mV)
Time (min)

Figure 2

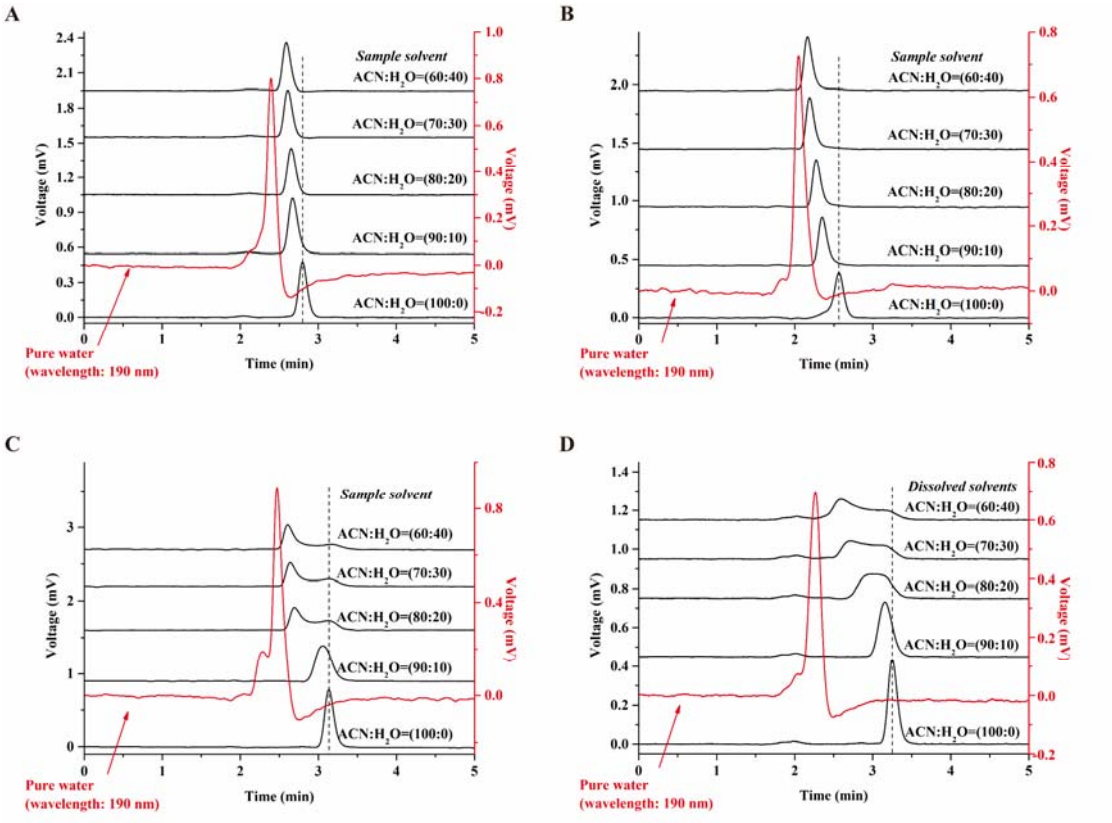

A
Sample solvent
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Pure water
(wavelength: 190 nm)
Voltage (mV)
Time (min)
B
Sample solvent
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Pure water
(wavelength: 190 nm)
Voltage (mV)
Time (min)
C
Sample solvent
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Pure water
(wavelength: 190 nm)
Voltage (mV)
Time (min)
D
Dissolved solvents
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Pure water
(wavelength: 190 nm)
Voltage (mV)
Time (min)

Figure 3

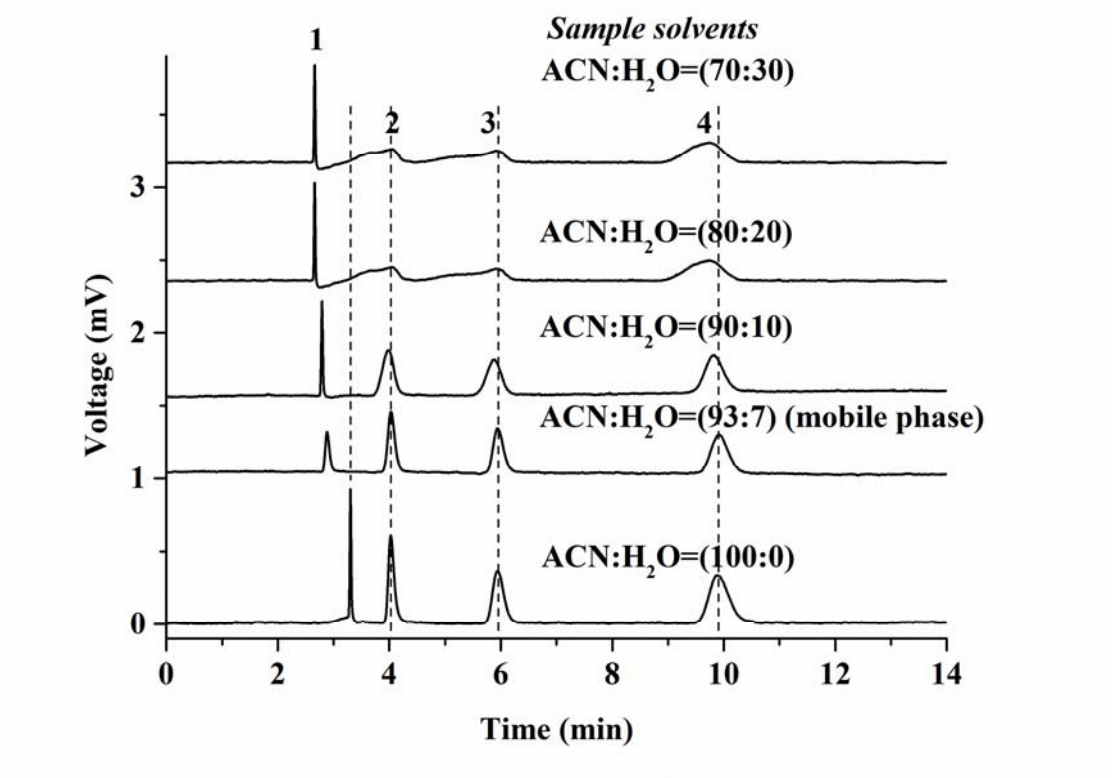

Sample solvents
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(93:7) (mobile phase)
ACN:H2O=(100:0)
1
2
3
4
Voltage (mV)
Time (min)
0
1
2
3
0
2
4
6
8
10
12
14

Figure 4

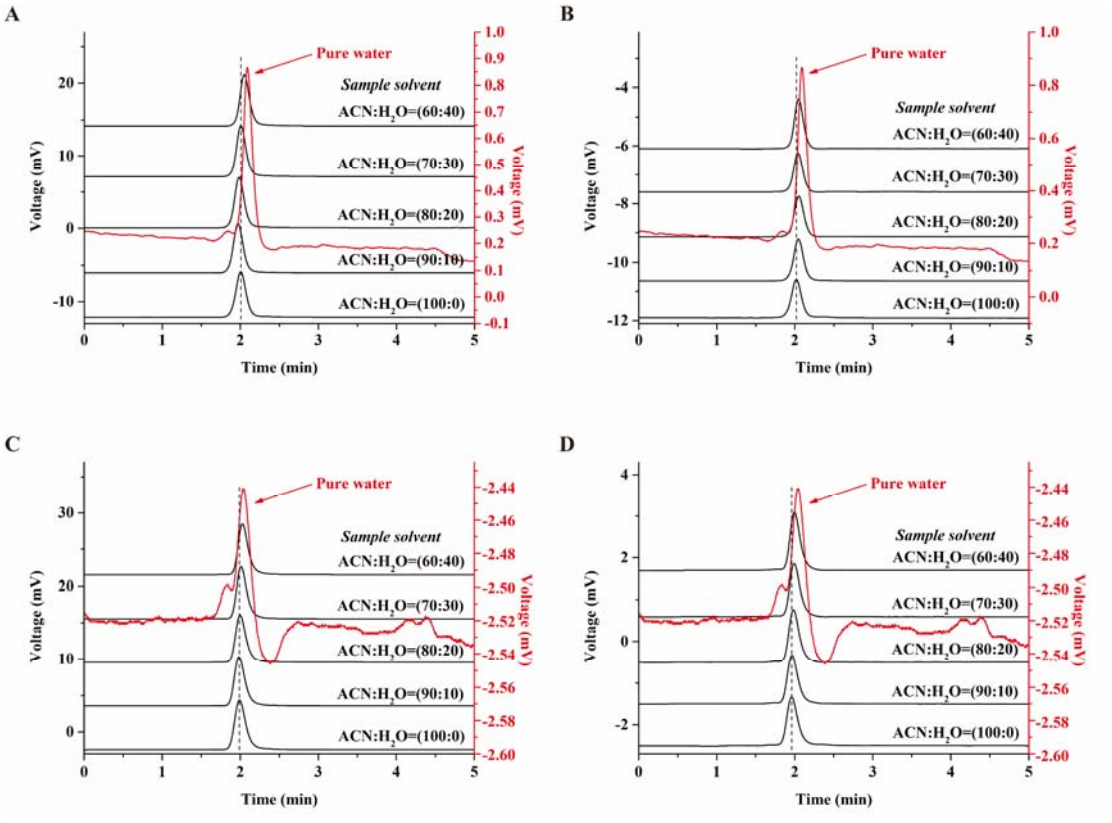

A
B
C
D
Pure water
Sample solvent
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Voltage (mV)
Time (min)

Figure 5

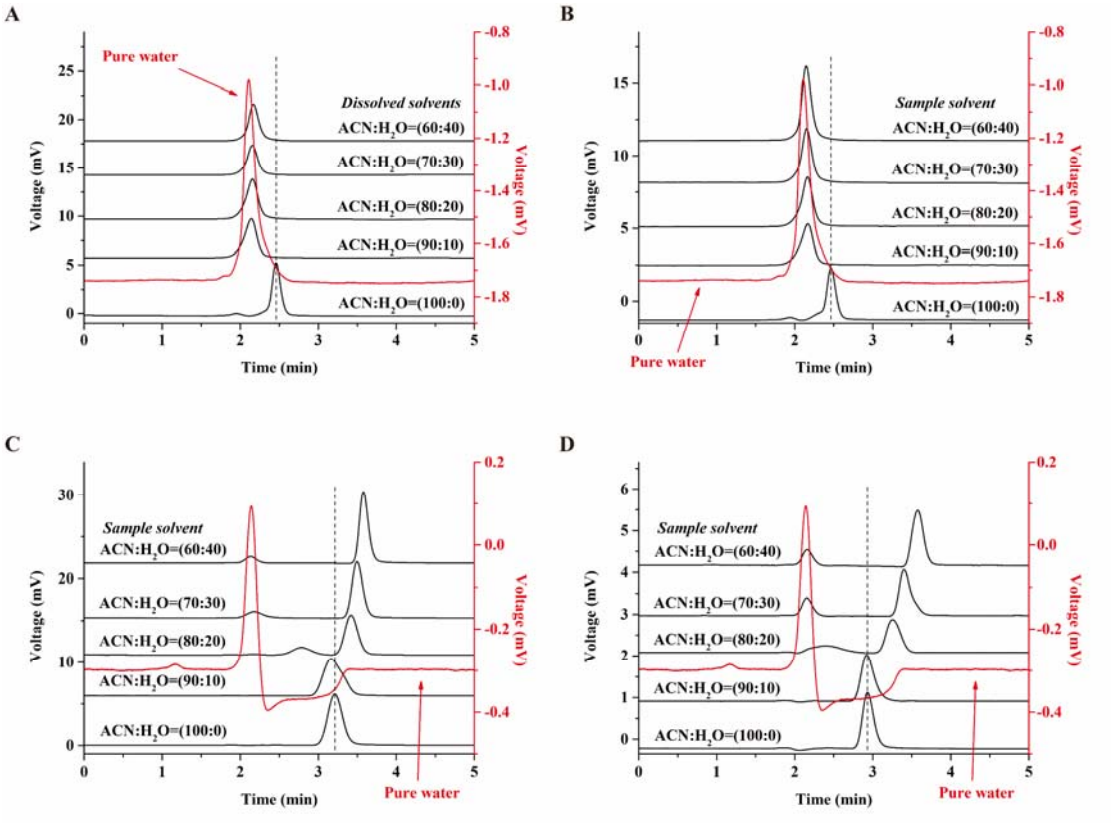

A
Pure water
Dissolved solvents
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Voltage (mV)
Time (min)
B
Sample solvent
Pure water
C
D

Figure 6

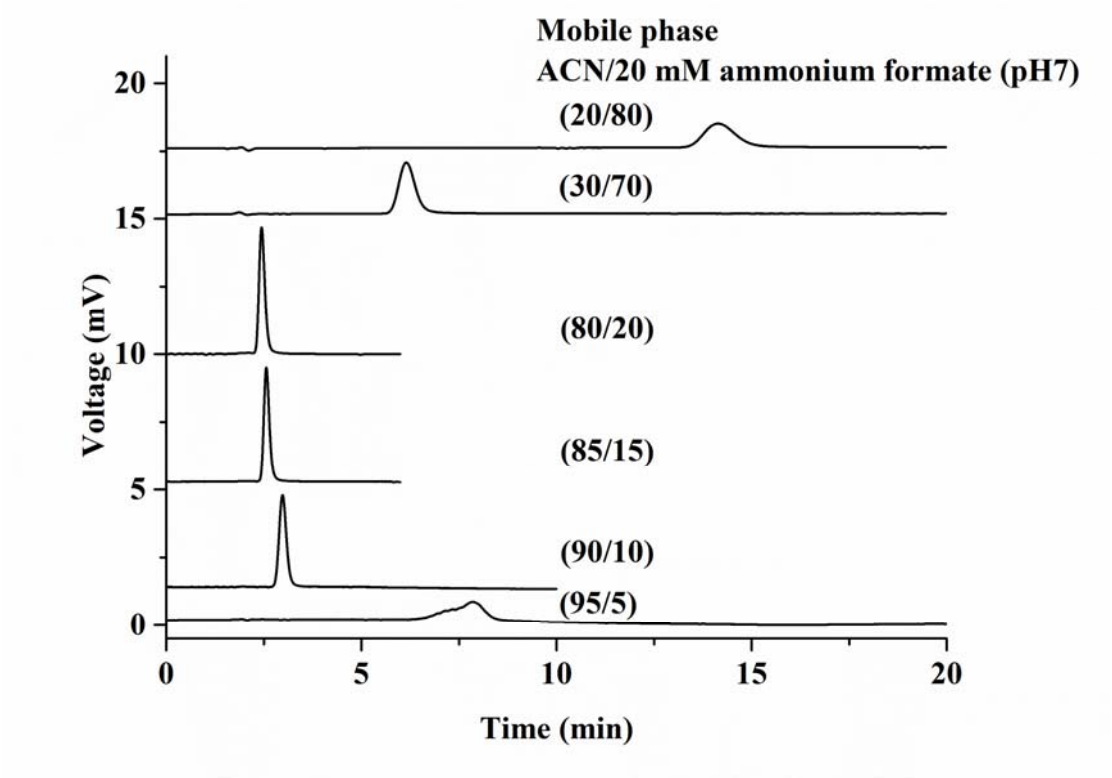

Mobile phase
ACN/20 mM ammonium formate (pH7)
(20/80)
(30/70)
(80/20)
(85/15)
(90/10)
(95/5)
Voltage (mV)
Time (min)
0
5
10
15
20

Figure 7

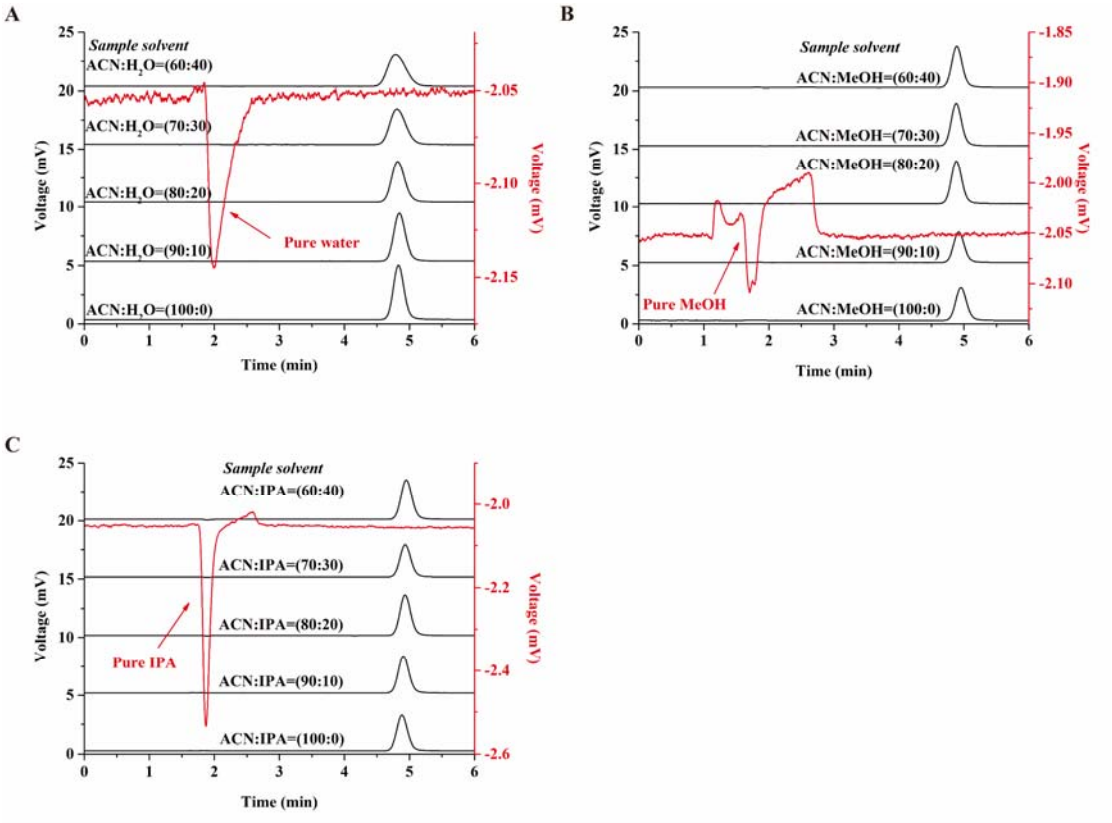

A
Sample solvent
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Pure water
Voltage (mV)
Time (min)
B
Sample solvent
ACN:MeOH=(60:40)
ACN:MeOH=(70:30)
ACN:MeOH=(80:20)
ACN:MeOH=(90:10)
ACN:MeOH=(100:0)
Pure MeOH
Voltage (mV)
Time (min)
C
Sample solvent
ACN:IPA=(60:40)
ACN:IPA=(70:30)
ACN:IPA=(80:20)
ACN:IPA=(90:10)
ACN:IPA=(100:0)
Pure IPA
Voltage (mV)
Time (min)

Figure 8

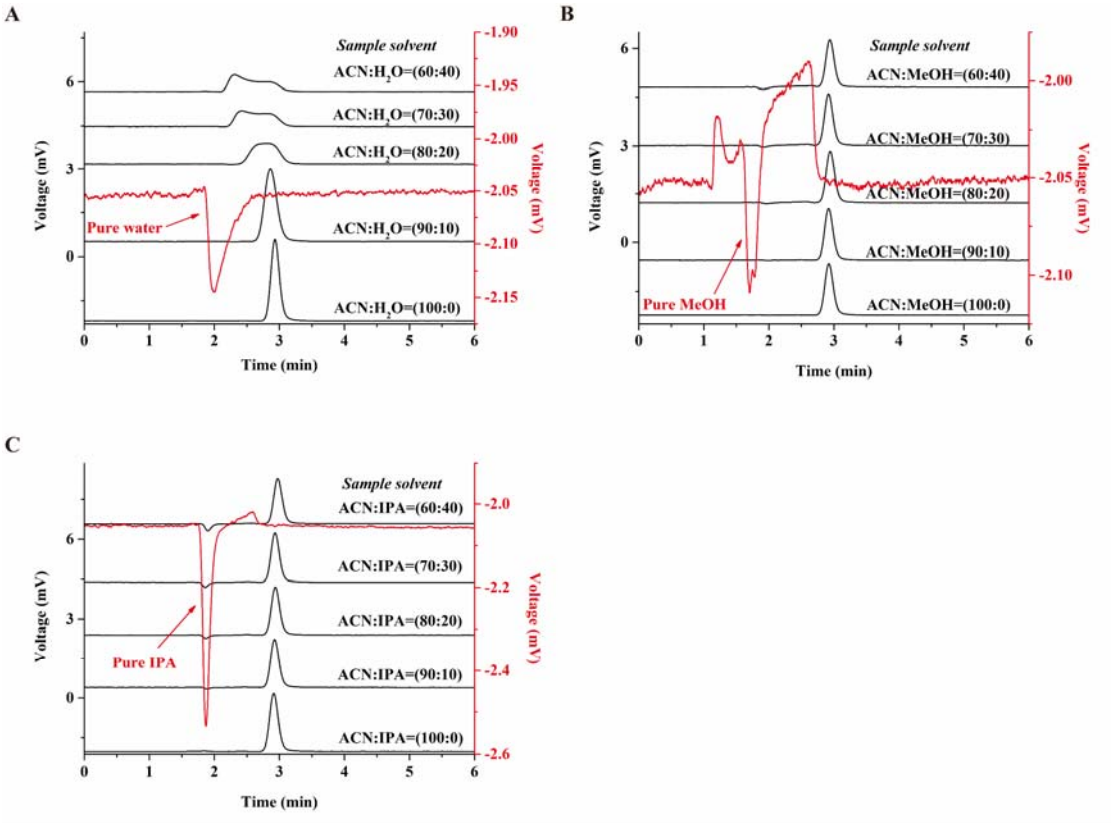

A
Sample solvent
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Pure water
Voltage (mV)
Time (min)
B
Sample solvent
ACN:MeOH=(60:40)
ACN:MeOH=(70:30)
ACN:MeOH=(80:20)
ACN:MeOH=(90:10)
ACN:MeOH=(100:0)
Pure MeOH
Voltage (mV)
Time (min)
C
Sample solvent
ACN:IPA=(60:40)
ACN:IPA=(70:30)
ACN:IPA=(80:20)
ACN:IPA=(90:10)
ACN:IPA=(100:0)
Pure IPA
Voltage (mV)
Time (min)

Figure 9

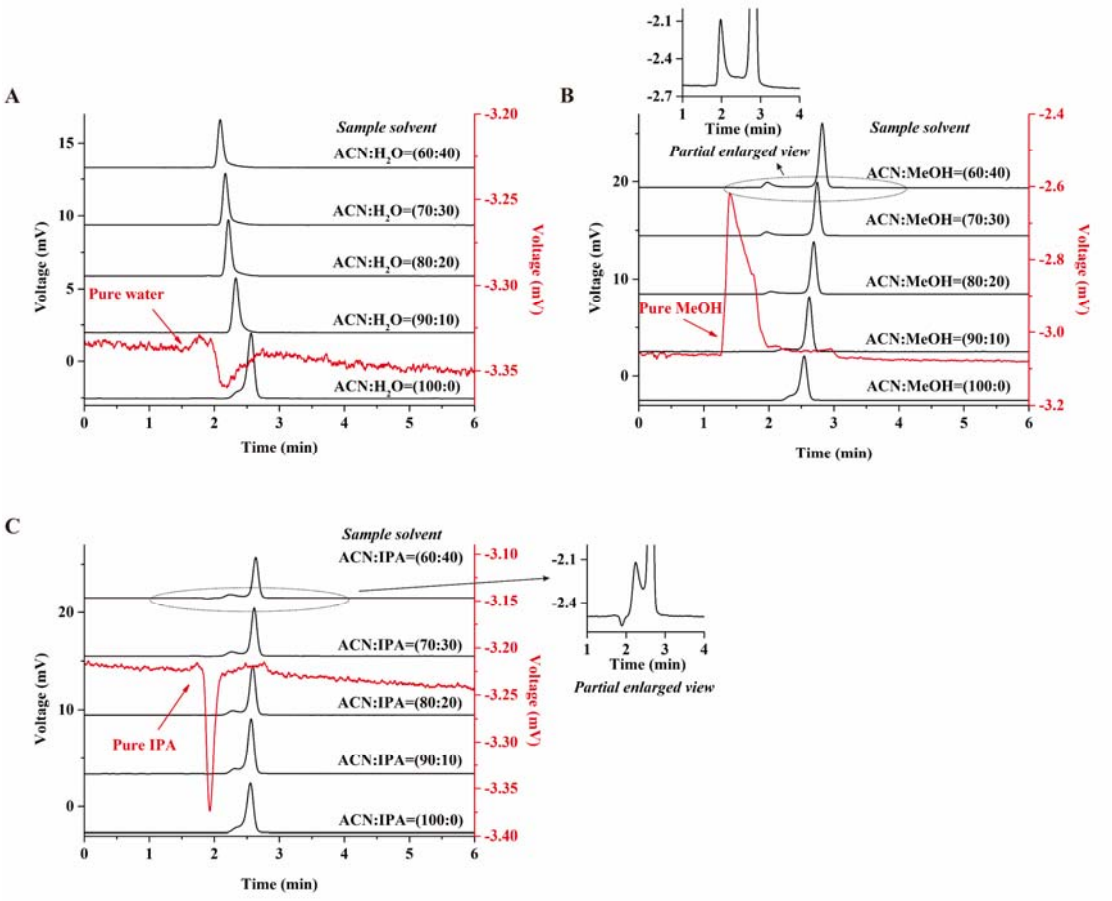

A
Sample solvent
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Pure water
Voltage (mV)
Time (min)
B
Sample solvent
Partial enlarged view
ACN:MeOH=(60:40)
ACN:MeOH=(70:30)
ACN:MeOH=(80:20)
ACN:MeOH=(90:10)
ACN:MeOH=(100:0)
Pure MeOH
Voltage (mV)
Time (min)
C
Sample solvent
ACN:IPA=(60:40)
ACN:IPA=(70:30)
ACN:IPA=(80:20)
ACN:IPA=(90:10)
ACN:IPA=(100:0)
Pure IPA
Partial enlarged view
Voltage (mV)
Time (min)

Figure 10

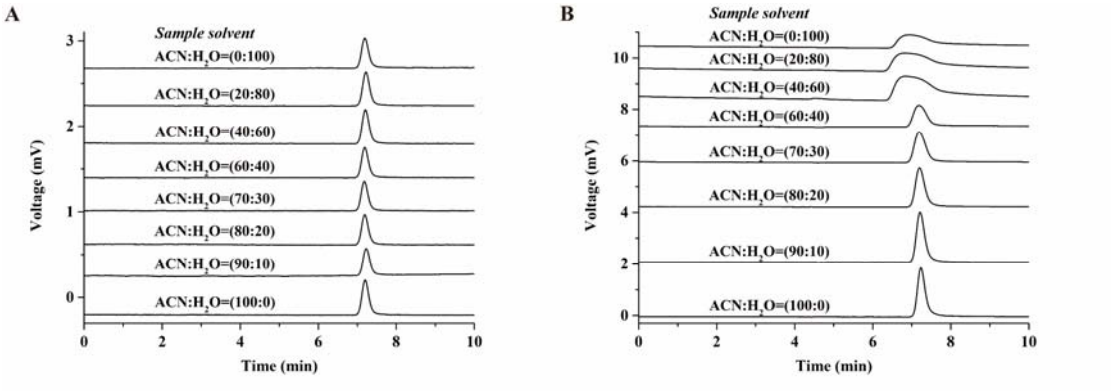
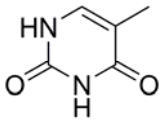
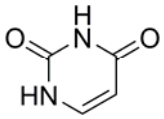
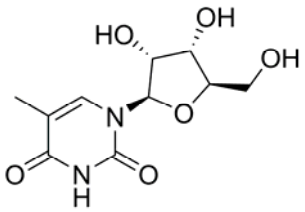
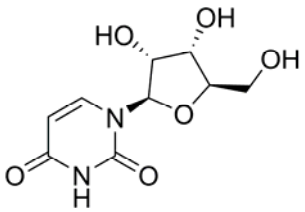
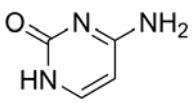
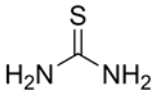
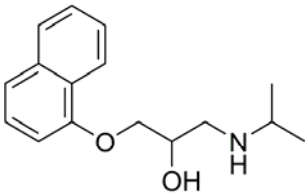
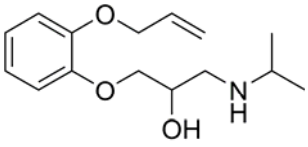

A
Sample solvent
ACN:H2O=(0:100)
ACN:H2O=(20:80)
ACN:H2O=(40:60)
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Voltage (mV)
Time (min)
B
Sample solvent
ACN:H2O=(0:100)
ACN:H2O=(20:80)
ACN:H2O=(40:60)
ACN:H2O=(60:40)
ACN:H2O=(70:30)
ACN:H2O=(80:20)
ACN:H2O=(90:10)
ACN:H2O=(100:0)
Voltage (mV)
Time (min)

Table 1. Physicochemical properties of the analytes. These properties were taken from the database of SciFinder (www.scifinder.cas.org)

Compound	logP	pKa	Molar Solubility in water (25 °C) (mol L^{-1}) (pH 7)	Formula
Thymine	-0.621±0.214	9.24±0.10	0.0072	
Uracil	-1.037±0.212	8.95±0.10	0.054	
5-Methyluridine	-1.166±0.322	9.55±0.10	0.024	
Uridine	-1.581±0.321	9.39±0.10	0.054	
Cytosine	-1.962±0.348	9.00±0.10	0.21	
Thiourea	-1.020±0.187	14.91±0.29	1.99	
Propranolol	2.900±0.247	9.50±0.30	0.52	
Oxprenolol	2.151±0.267	9.44±0.10	3.77	

Supporting Information

A systematic investigation of the effect of sample solvent on peak shape in nano- and microflow hydrophilic interaction liquid chromatography columns

Haibin Li [a,b,c], Chusheng Liu [d], Li Zhao [a,b], Dongsheng Xu [a,b], Tingting Zhang [a,b], Qiqin Wang [a,b], Deirdre Cabooter [c*], Zhengjin Jiang [a,b*]

[a] *Institute of Pharmaceutical Analysis, College of Pharmacy, Jinan University, Guangzhou 510632, China*

[b] *International Cooperative Laboratory of Traditional Chinese Medicine Modernization and Innovative Drug Development of Chinese Ministry of Education (MOE), College of Pharmacy, Jinan University, Guangzhou 510632, China*

[c] *KU Leuven, Department for Pharmaceutical and Pharmacological Sciences, Pharmaceutical Analysis, Herestraat 49, 3000 Leuven, Belgium*

[d] *NMPA Key Laboratory for Monitoring and Evaluation of Cosmetics, Shenzhen Institute for Drug Control, Shenzhen 518057, China*

[*] Corresponding authors:

E-mail address: deirdre.cabooter@kuleuven.be (D. Cabooter), jzjjackson@hotmail.com (Z. Jiang)

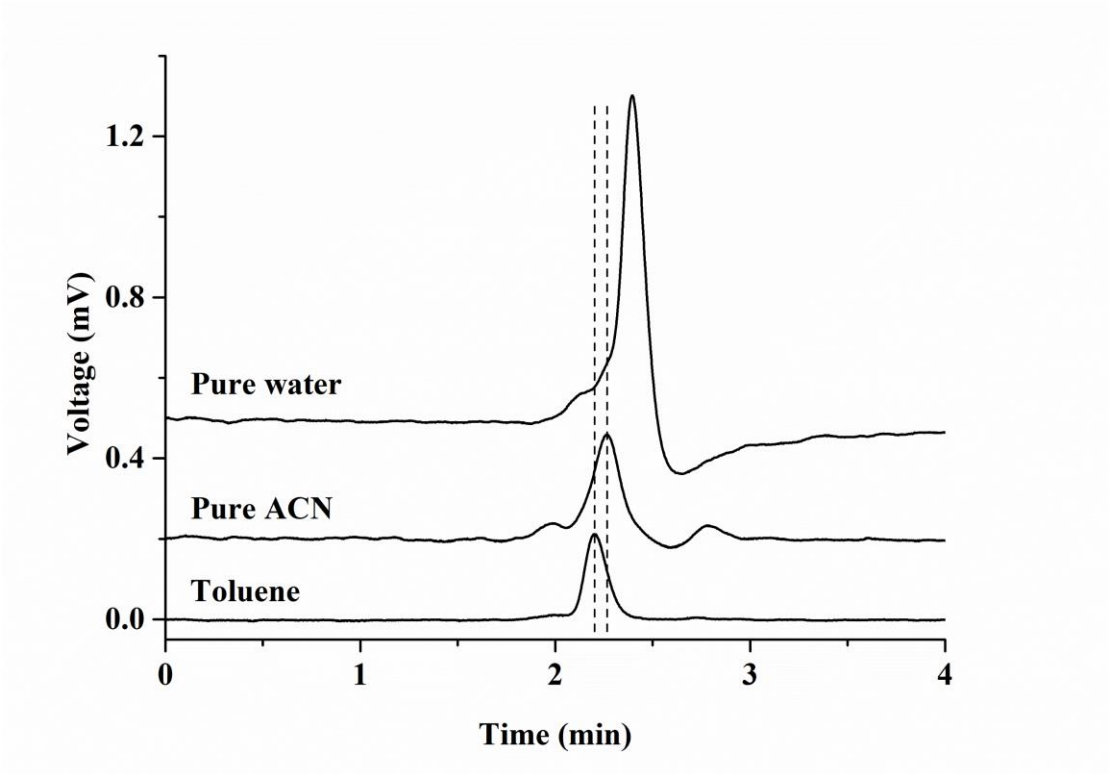


Figure S1. Chromatograms showing the elution time of pure water (H_2O), pure acetonitrile (ACN) and toluene on a poly(SPDA-co-MBA) monolithic column; isocratic mobile phase consisting of ACN:H_2O (93:7); detection wavelength: 190 nm for pure water and pure ACN and 254 nm for toluene. The vertical dashed lines indicate the peak maximum of the toluene and ACN peak, respectively.

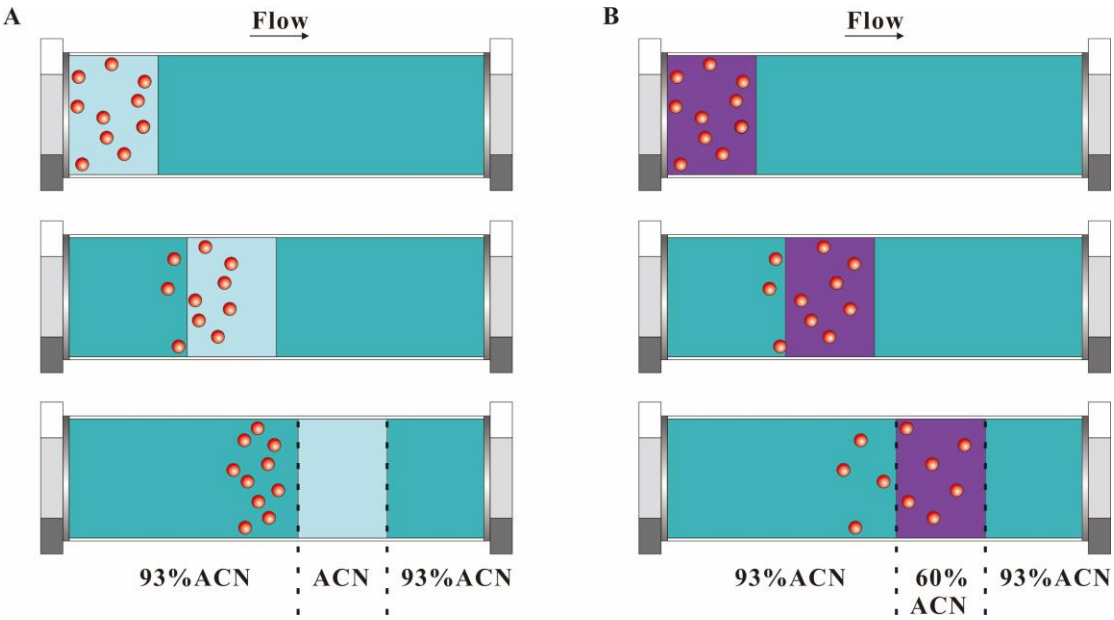


Figure S2. Simulated representations of the solvent zone in the column when using ACN:H_2O (93:7, v:v) as the mobile phase. A) Sample solvent: ACN, the sample peak is narrow because of the weak elution capacity of ACN, B) Sample solvent: ACN:H_2O (60:40, v:v), the analyte band is wider due to the strong elution strength of the highly aqueous sample solvent.

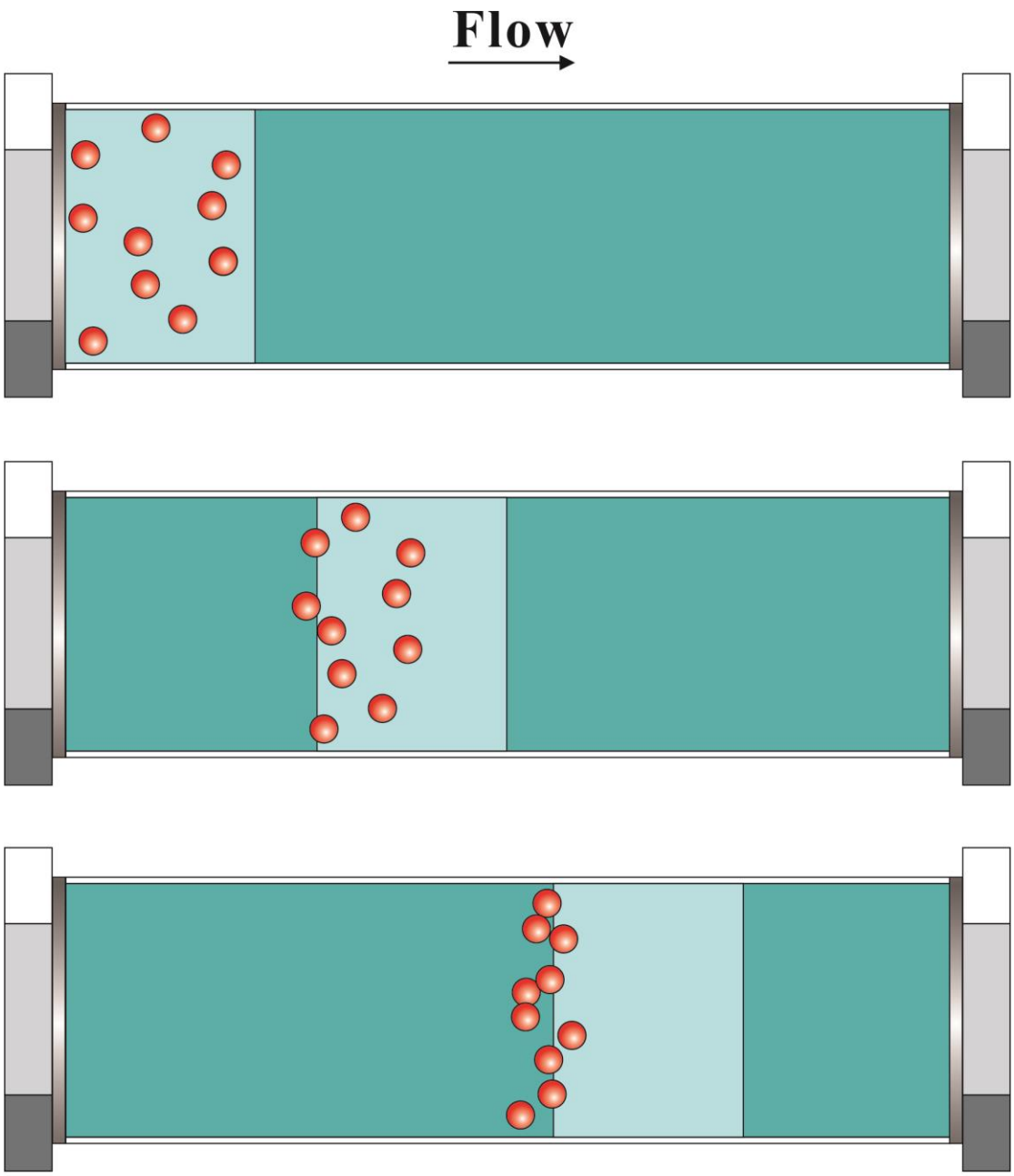


Figure S3. Simulated representation of the solvent zone in the column explaining the observed elution behavior of thymine. The elution strength at the front of the analyte band (dissolved in mobile phase) will always be slightly weaker than that at the back of the analyte band (dissolved in ACN).

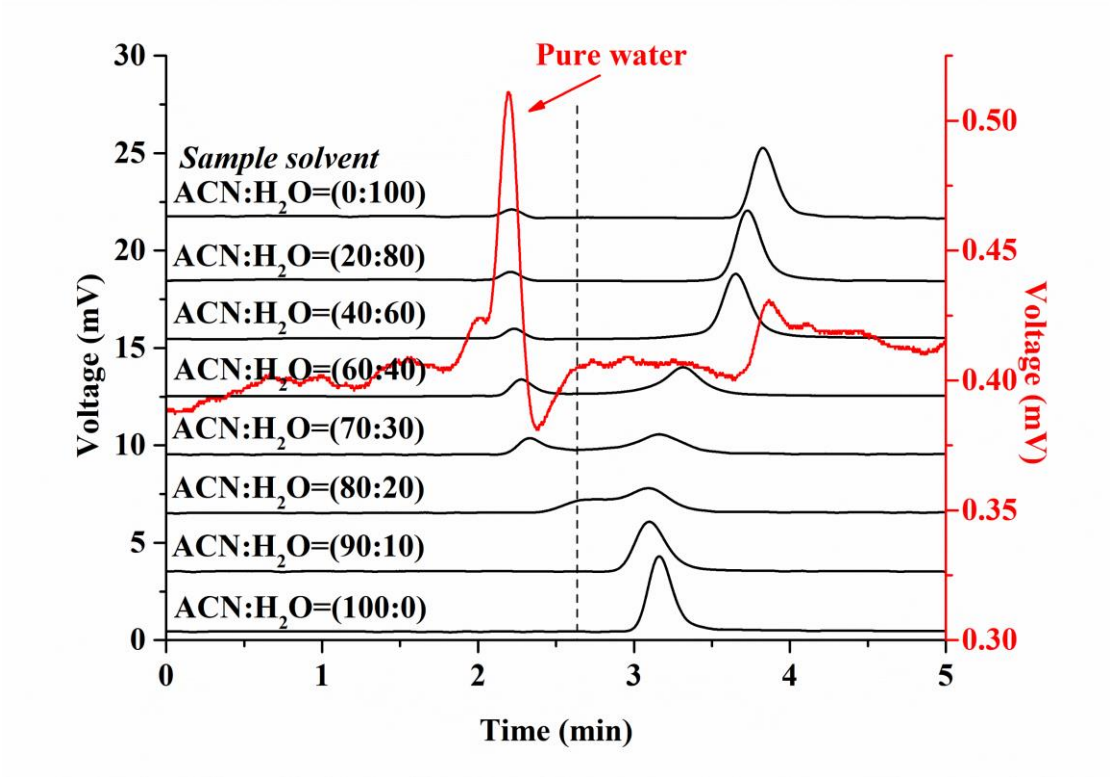


Figure S4. Effect of sample solvent composition on a charged compound eluting after the solvent peak. Experimental conditions: column: poly(SPA-co-MBA) monolithic column; detection wavelength: 230 nm; mobile phase: ACN:20 mM ammonium formate (pH 7.0) (90:10, v:v); sample: propranolol.

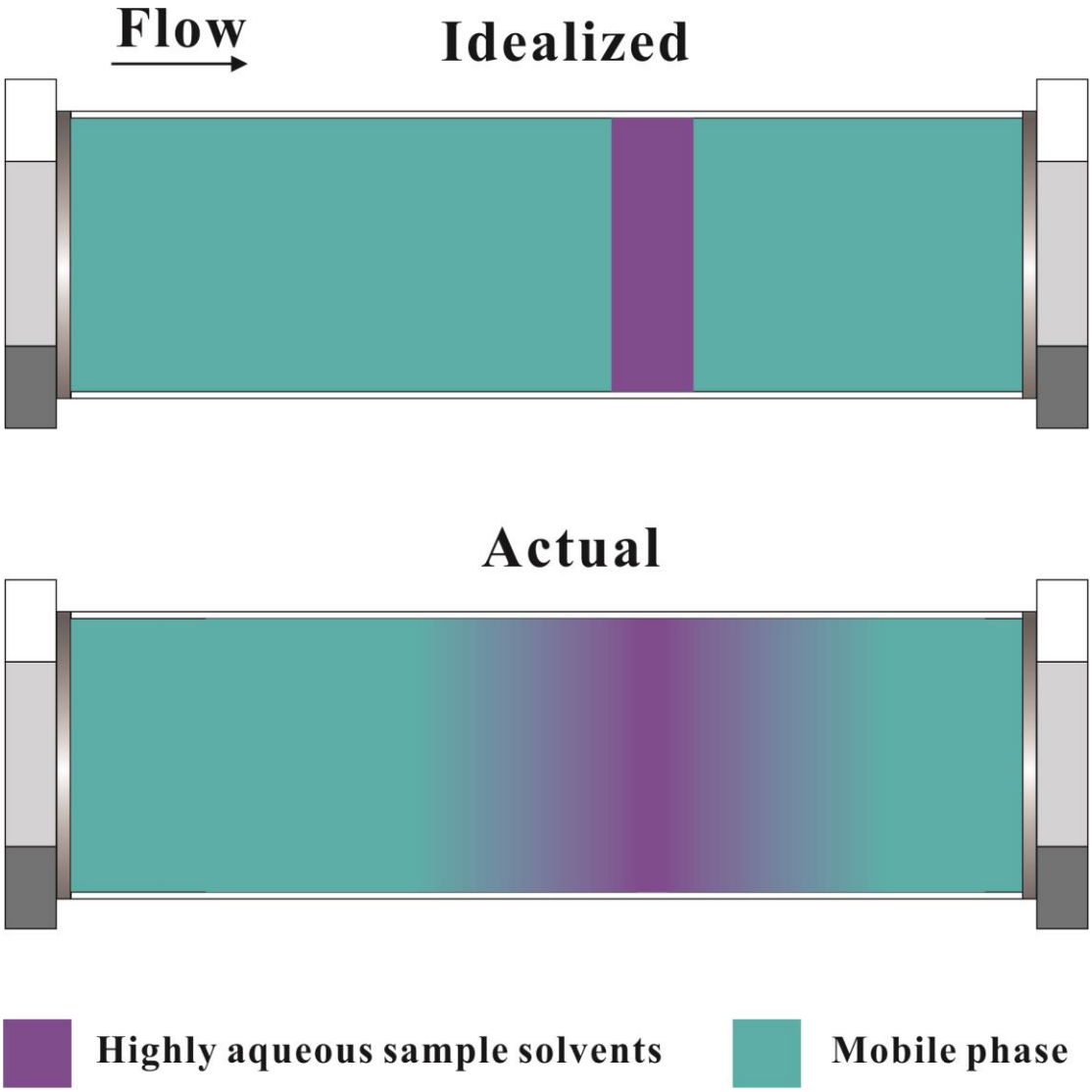


Figure S5. Simulated representations of the solvent zone in the column when highly aqueous sample solvents move through the column.

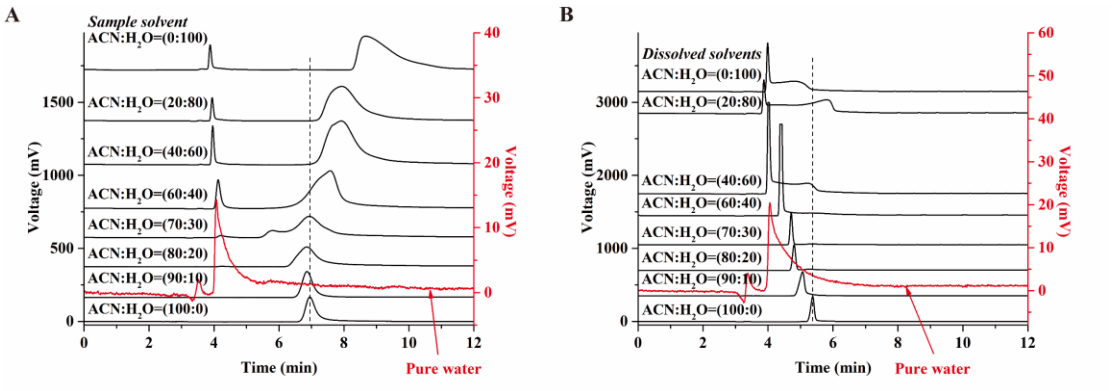


Figure S6. Effect of the sample solvent on two zwitterionic silica columns (ZIC-HILIC & ZIC-cHILIC). Experimental conditions: injection volume: 0.2 µl; detection wavelength: 230 nm; mobile phase: ACN:20 mM ammonium formate (pH 4.0) (90:10, v:v); sample: propranolol; column: A) ZIC-HILIC, B) ZIC-cHILIC.

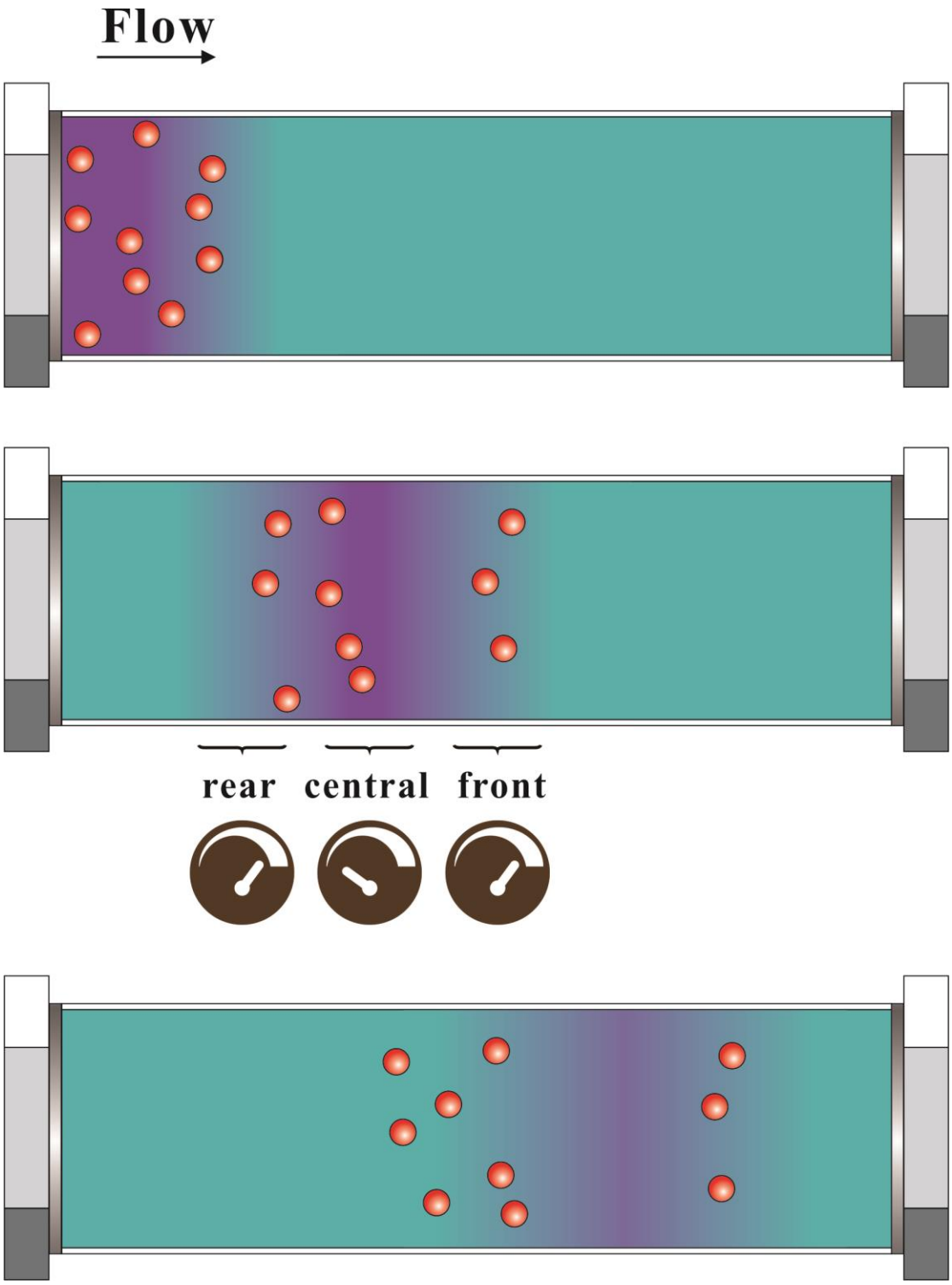


Figure S7. Simulated representations of the process of the breakthrough peak in ZIC-HILIC. The water content in the front part of the solvent band was always low, so the elution strength was high.

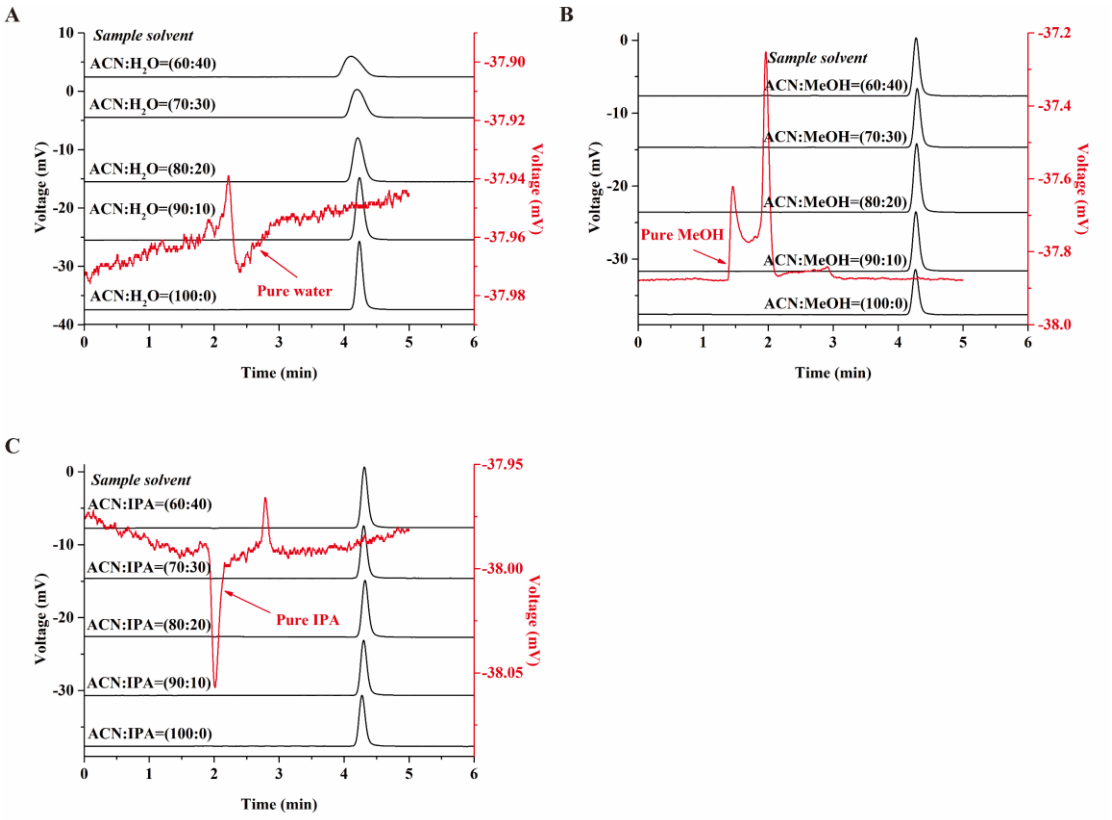


Figure S8. Effect of MeOH and IPA in the sample solvent on the resulting peak shape of thiourea (k= 1.12). Experimental conditions: column: poly(SPP-co-MBA) monolithic column; detection wavelength: 254 nm; mobile phase: ACN:H_2O (93:7, v:v).

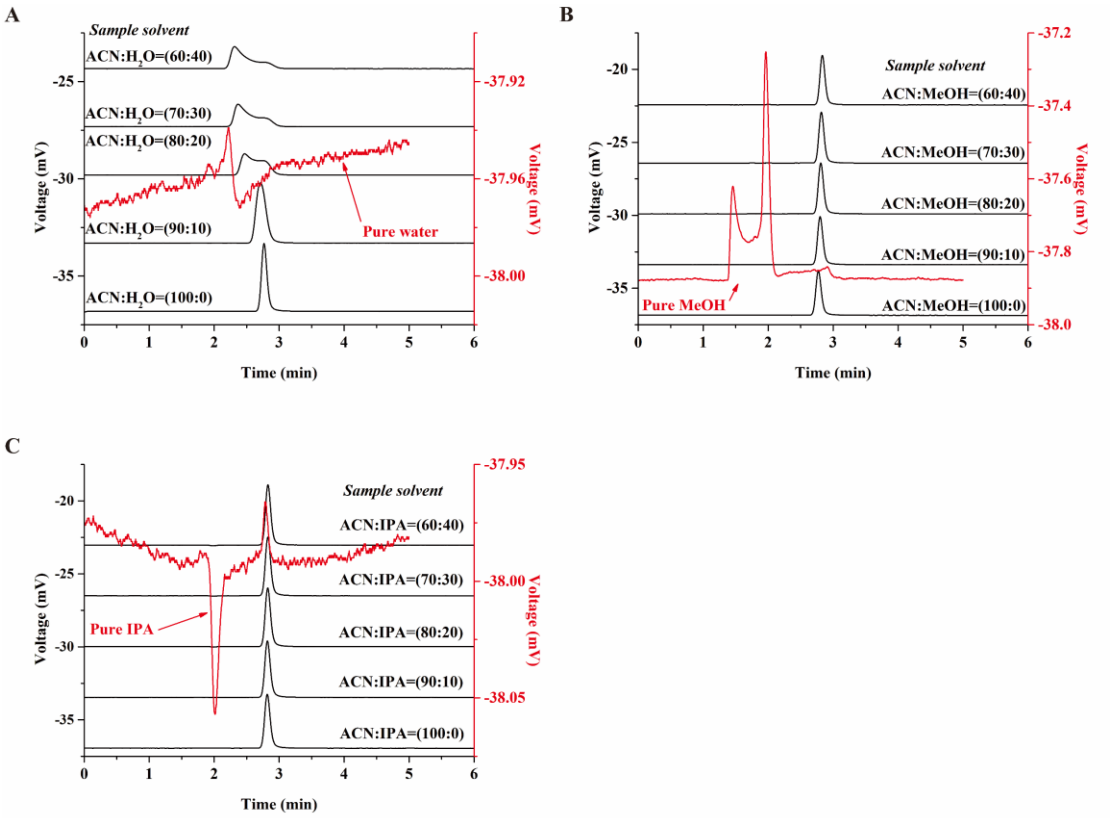


Figure S9. Effect of MeOH and IPA in the sample solvent on the resulting peak shape of uracil (k= 0.37). Experimental conditions: column: poly(SPP-co-MBA) monolithic column; detection wavelength: 254 nm; mobile phase: ACN:H_2O (93:7, v:v).

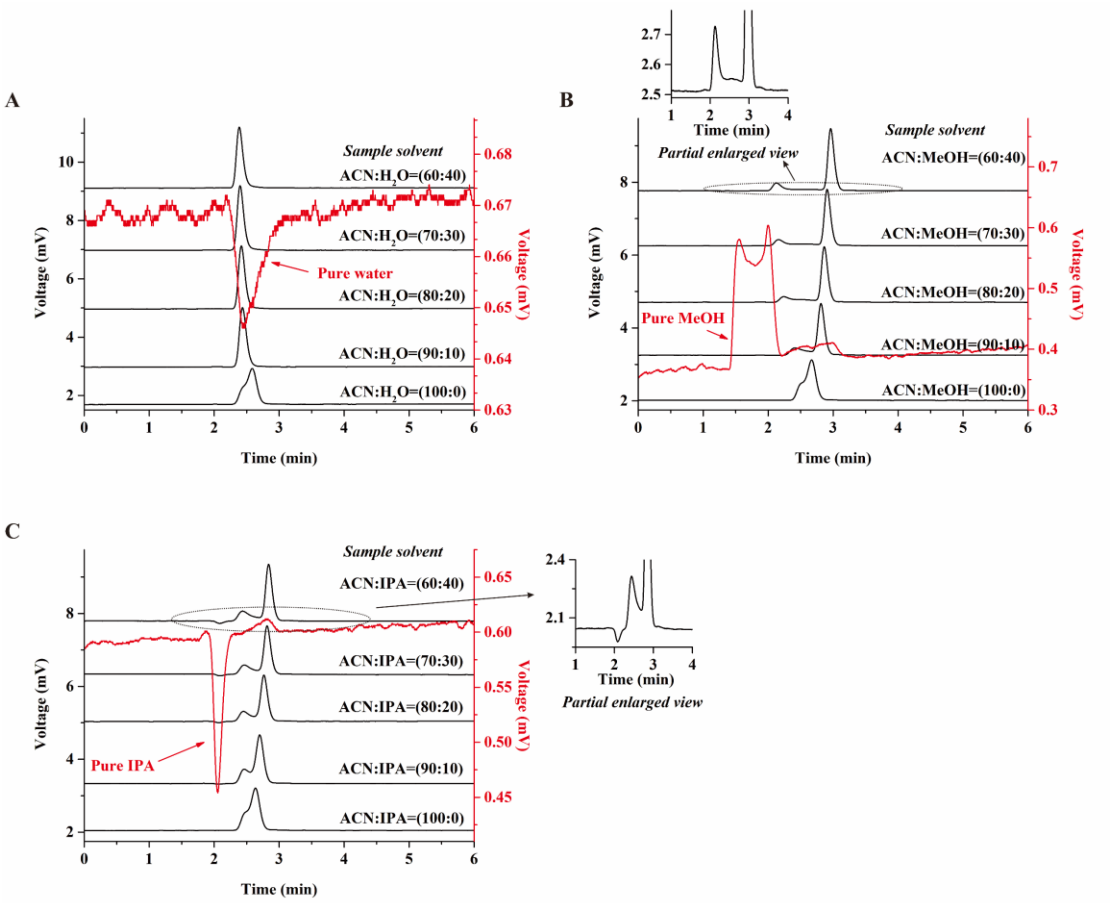


Figure S10. Effect of MeOH and IPA in the sample solvent on the resulting peak shape of thymine (k= 0.29). Experimental conditions: column: poly(SPP-co-MBA) monolithic column; detection wavelength: 254 nm; mobile phase: ACN:H_2O (93:7, v:v).