CHEMIJA. 2017. Vol. 28. No. 4. P. 233–239

Effect of sample solvents on the peak shape of small polar compounds in hydrophilic interaction chromatography

Inga Baškirova,

Giedrė Rušėnaitė,

Vilma Olšauskaitė,

Audrius Padarauskas*

Department of Analytical and Environmental Chemistry, Faculty of Chemistry and Geosciences, Vilnius University, Naugarduko St. 24, 03225 Vilnius, Lithuania

The effect of various sample diluents and the injection volume on the peak shapes of small polar compounds (benzoic acid, melamine, nicotine and nicotinic acid) on the two stationary phases (bare silica and amide-bonded silica) was evaluated under hydrophilic interaction chromatography conditions. Seven solvents tested include water, acetonitrile, methanol, ethanol, isopropanol, dimethyl sulfoxide and ethyl acetate. Using the amide phase acceptable peak shapes were obtained in acetonitrile, ethyl acetate, ethanol and isopropanol. In the case of the bare silica phase only acetonitrile and ethyl acetate provided adequate peak efficiencies for all analytes. Higher resistance to a sample diluent of the amide phase may be attributed to its less hydrophilic character. The maximal sample volume that does not cause peak broadening depends on the nature of the sample diluent. When the content of water in acetonitrile increased, peaks were broadened or even distorted. Acceptable peak widths of the most strongly retained nicotinic acid were obtained when the injected sample contained ≤40% or ≤60% water for the bare silica and the amide phase, respectively. For the three less retained analytes, depending on a particular analyte and stationary phase, the critical amount of water in acetonitrile varied between 20 and 30%.

Keywords: hydrophilic interaction chromatography, sample solvent, peak shape

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 liquid 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].

Before a sample is injected into an LC system, it must be dissolved into a suitable diluent. If the sample solvent has properties different from those of the mobile phase, the result can range from slight broadening to extreme distortions or even fragmentation of peaks. One possible cause for these effects is a difference in the elution strength between the diluent and the mobile phase. Peak broadening and shape abnormalities generally get worse as the diluent becomes stronger than the mobile phase [7, 8]. Another possible reason for peak distortions is a hydrodynamic instability which occurs at the boundary of the diluent and the mobile phase, caused by the difference in viscosity between the two liquids [9, 10]. Regardless of the cause, the simplest and most obvious solution to this problem is using the mobile phase itself as a diluent, thereby eliminating any strength or viscosity mismatch between the two. However, this is not always practical due to other considerations such as chemical stability and sample solubility.

*Corresponding author. Email: audrius.padarauskas@chf.vu.lt

Significant peak distortion caused by sample diluent solvents occurs in all modes of chromatography. For RP-LC, the effects of sample solvents have been well documented [11]. However, there are only a few studies that reported sample solvent effects in the HILIC separation mode [12, 13].

The aim of the present work was to expand upon the previous studies of peak broadening and distortion as a function of sample solvent nature in HILIC. The effect of various sample diluents and the injection volume on peak shapes of small ionizable compounds on the two most popular HILIC stationary phases (bare silica and amide-bonded silica) was evaluated.

EXPERIMENTAL

Ultra-pure water was obtained from a Mili-Q Water Purification System from Millipore (Bedford, MA, USA). Acetonitrile (ACN), dimethyl sulfoxide (DMSO), ethanol (EtOH), ethyl acetate (EtOAc), methanol (MeOH), isopropanol (IPA), acetic acid and ammonium acetate were of LC-MS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Benzoic acid (≥99%), melamine (≥98%), nicotine (≥99%) and nicotinic acid (≥98%) were also from Sigma-Aldrich.

Individual stock solutions of analytes at a concentration of 500 mg/L were prepared in an ACN/H_2O (1:1, v/v) solution containing a 25 mmol/L ammonium acetate buffer (pH 5). Working standard solutions at 10 mg/L were prepared by diluting the stock solution with an appropriate solvent. Thus, the quantity of the solvent used for the preparation of the stock solution was negligible.

HILIC separations were performed on a Waters Acquity UPLC system (Waters, Milford MA, USA) equipped with an Acquity UPLC photodiode array detector (PDA). Acquity UPLC BEH HILIC (bare silica) and Acquity UPLC BEH Amide (silica coated with amide functional groups) columns (2.1 × 100 mm, 1.7 µm, Waters) were used in the experiments. The column temperature was maintained at 30°C. The mobile phase flow rate was 0.5 mL/min. The injection volume was 2 µL unless otherwise stated. The number of theoretical plates (*N*) was calculated using the half height method. Toluene was used as a void time marker. All results were the mean of duplicate injections. Data collection and management was performed by the Data Analysis 4.0 software (Bruker).

RESULTS AND DISCUSSION

Characterization of the analytes and solvents

We selected four polar compounds as model analytes to examine the effect of a sample solvent on their peak shapes. Their chemical structures are presented in Fig. 1. The pK_a and log *P* values of the analytes are listed in Table 1. The separation was performed under isocratic elution conditions employing the ACN/H_2O (94:6, v/v) mobile phase containing a 10 mmol/L ammonium acetate buffer (pH 5). Figure 2 shows the chromatograms obtained for the two investigated stationary phases. As expected, for acidic compounds the more hydrophilic analyte with a lower log *P* value (i.e. nicotinic acid) on both stationary phases shows a greater retention. The elution order of bases (melamine and nicotine) on the bare silica stationary phase was the opposite of that on the amide phase. Such behaviour was most likely due to a significant impact of the secondary cation-exchange interactions between negatively charged silanols of the bare silica surface and cationic analytes. Because nicotine exhibits a higher positive charge at pH 5 (see Table 1), it is more strongly affected by ion-exchange and, consequently, is stronger retained than less charged melamine. The chemically bonded amino phase does not contain negatively charged silanol groups other than unreacted residual silanols. Thus, the impact of ion-exchange on the retention of cationic compounds on this phase is negligible and they are retained mainly by partitioning.

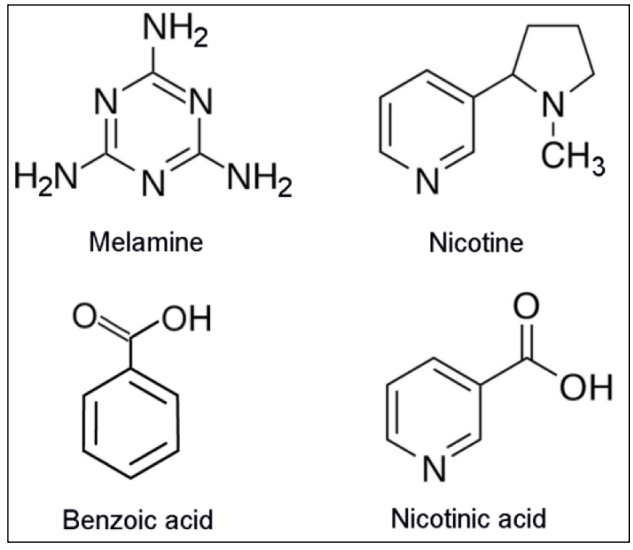

Fig. 1. Structures of the model analytes

Table 1. **Properties of model analytes [14]**

Analyte	pK_a	log P
Melamine (MEL)	pK_a = 5.0	–1.14
Nicotine (NIC)	pK_{a1} = 3.1 pK_{a2} = 8.0	1.17
Benzoic acid (BA)	pK_a = 4.2	1.87
Nicotinic acid (NA)	pK_a = 2.2 (acidic) pK_a = 4.8 (basic)	0.36

Seven solvents with varying polarity were selected as sample diluents for this study. As already mentioned in the introduction, two main properties of the sample solvent, namely, elution strength and viscosity, must be considered to ensure reliable peak shapes. In addition, under

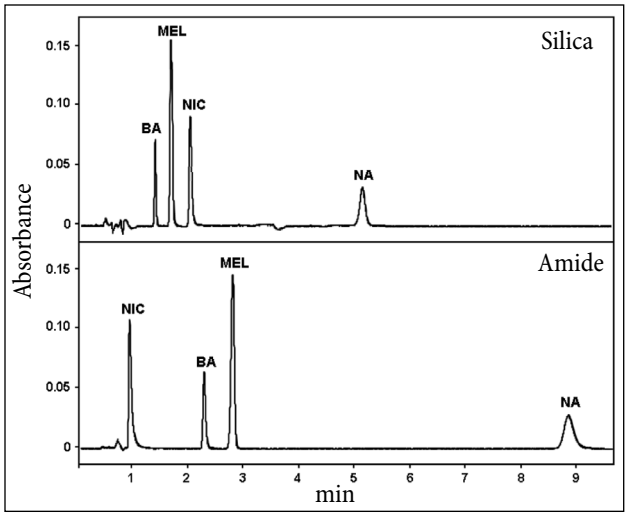

Fig. 2. Chromatograms of the model analytes obtained for two HILIC stationary phases. The mobile phase contains 10 mmol/L ammonium acetate buffer (pH 5) in ACN/H_2O (94:6, v/v). The flow rate is 0.5 mL/min. The injected volume is 2 µL. A sample diluent is a mobile phase

HILIC conditions the hydrogen bonding donor ability of the solvent may also contribute to peak broadening [13]. These properties of all the considered solvents are listed in Table 2. However, the elution strength data for the amide phase were not available.

Table 2. **Properties of solvents used in this study [15]**

Solvent	Elution strength ε° (silica)	Viscosity, cP	Hydrogen bonding properties
H_2O	≫1	1.00	Protic
Methanol (MeOH)	0.73	0.55	Protic
Ethanol (EtOH)	0.68	1.20	Protic
Isopropanol (IPA)	0.63	2.40	Protic
Acetonitrile (ACN)	0.52	0.38	Aprotic
Dimethyl sulfoxide (DMSO)	0.50	2.24	Aprotic
Ethyl acetate (EtOAc)	0.48	0.45	Aprotic

Sample solutions in pure solvents

Figures 3 and 4 present the peak profiles of four test analytes that were measured on both stationary phases. The only variation was the solvent used as a sample diluent. The elution strength of solvents increases in HILIC in the order EtOAc < DMSO < ACN < IPA < EtOH < MeOH < H_2O, as described by the ε° parameters calculated for bare silica (see Table 2). Even the visual inspection of the peaks shows significant differences. In general, as the solvent elution strength increases, peak broadening and distortion increase. In addition, broader peaks were observed using protic solvents. This was true for all the diluents with the exception of

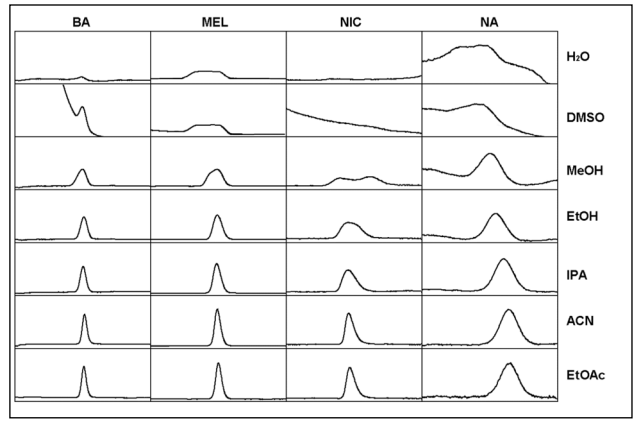

Fig. 3. The effect of different sample solvents on peak profiles of test analytes obtained on the bare silica stationary phase

DMSO. Because of their strong ability to form a hydrogen bond, polar protic solvents might disturb a water layer at the stationary phase surface [13]. As a consequence, peaks may be additionally broadened, distorted or even disappear. As expected, the most significant peak shape abnormalities occurred for the water diluent which exhibits the highest elution strength and the strongest hydrogen bonding donor ability.

Surprisingly, despite its weak elution strength and aprotic nature, DMSO on both phases produced entirely unsatisfactory peaks for most analytes. A similar anomalous behaviour of DMSO was also observed in earlier studies [13, 16]. We suspected that such behaviour may be attributed to a strong absorbance of DMSO in the UV range, but poor peak shapes were also obtained when mass spectrometric detection was employed (results not shown). Another possible cause of poor peak shapes might be viscosity effects. The viscosity of DMSO differs by more than 5 times from the acetonitrile rich mobile phase (see Table 2). According to viscosity fingering theory, peak distortion increases when the mismatch in viscosity of the mobile phase and the sample diluent becomes larger [17]. The fluid with

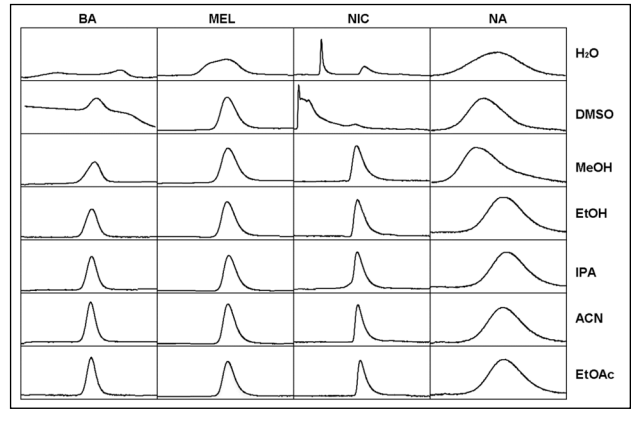

Fig. 4. The effect of different sample solvents on the peak profiles of test analytes obtained on the amide stationary phase

lower viscosity tends to penetrate into the other fluid creating an important distortion of the elution band, leading to the loss of peak efficiency. However, a detailed investigation of viscosity effects is beyond the scope of this report.

Retention factors (*k*) and the number of theoretical plates (*N*) obtained for the test analytes on both stationary phases employing different solvents are presented in Tables 3 and 4. As can be observed, the variation of the retention factors between solvents was negligible. The largest percentage of variation (~3.4%) was obtained for the most strongly retained nicotinic acid.

On both stationary phases ACN and EtOAc provided the highest peak efficiency for all analytes. Thus, these two solvents are best suited as sample diluents under the HILIC conditions employed in the present study. In addition, it can be noted that the most strongly retained analyte (nicotinic acid) was less prone to peak broadening than other analytes, similarly to what can be observed in RP-LC [10]. For example, its peak efficiency on the amide phase remained acceptable even in the DMSO diluent (*N* decreases from 15130 for ACN to 14170 for DMSO), whereas the peak of the least retained nicotine in the DMSO diluent completely disappeared. This proved that when the interactions between analyte and stationary phase are strong, the sample solvent is not able to influence the shape of the analyte elution band anymore.

Finally, it is important to note that at least for the test analytes, the sample solvent seems to be less critical for the amide stationary phase than for the bare silica one. According to the obtained results, using the amide phase acceptable peak efficiencies were obtained with EtOAc, ACN, IPA and EtOH, although the latter produced about 35% efficiency reduction for benzoic acid. In the case of the bare silica phase only EtOAc and ACN provided adequate peak efficiencies for all analytes. Higher resistance to the sample diluent of the amide phase compared to that of the bare silica may be attributed to its less hydrophilic character.

Influence of the sample volume

The simplest and the most straightforward way to enhance detection sensitivity is the increase of the injected sample volume. However, injection of excessive sample volumes leads to peak broadening and a loss of sensitivity. The maximal sample volume that does not cause peak broadening depends on the nature of the sample diluent. In this study, the effect of injection volume (1.0–7.5 µL) on the peak shapes of the test analytes dissolved in the four best suited solvents (EtOAc, ACN, IPA and EtOH) was examined. As an example, Fig. 5 shows the peak profiles of nicotine measured on both stationary phases. As is apparent from the peak profiles, the sample volume at a given diluent can also have a drastic effect on the peak shape. Table 5 summarizes the percent increase in the peak width at half height induced by injecting 1.0–7.5 µL solutions of the analytes dissolved in the four solvents using two stationary

Table 3. **The effect of the sample solvent on retention factors (*k*) and the number of theoretical plates (*N*) for test analytes (bare silica stationary phase)**

Solvent	BA		MEL		NIC		NA	
	k	*N*	*k*	*N*	*k*	*N*	*k*	*N*
H_2O	–[a]	–	2.12	562	–	–	–	–
DMSO	1.57	2332	2.13	482	–	–	8.99	5553
MeOH	1.58	3866	2.14	2565	–	–	9.06	11583
EtOH	1.60	6864	2.14	6430	3.07	2145	9.18	12970
IPA	1.60	9541	2.14	7602	3.05	5423	9.21	13530
ACN	1.62	14200	2.15	12485	3.06	11640	9.31	14821
EtOAc	1.61	14108	2.16	14095	3.07	11249	9.31	14830

[a] Split or distorted peak.

Table 4. **The effect of a sample solvent on retention factors (*k*) and the number of theoretical plates (*N*) for test analytes (amide stationary phase)**

Solvent	BA		MEL		NIC		NA	
	k	*N*	*k*	*N*	*k*	*N*	*k*	*N*
H_2O	–[a]	–	4.61	4230	–	–	17.35	7580
DMSO	3.81	8350	4.70	10950	–	–	17.28	14170
MeOH	3.81	8400	4.72	10742	1.06	1894	17.23	13880
EtOH	3.78	10040	4.76	12940	1.05	2394	17.42	14580
IPA	3.77	13280	4.75	13663	1.08	3809	17.58	14820
ACN	3.75	15890	4.78	14495	1.08	4312	17.54	15130
EtOAc	3.76	15610	4.78	14560	1.07	4380	17.50	15095

[a] Split or distorted peak.

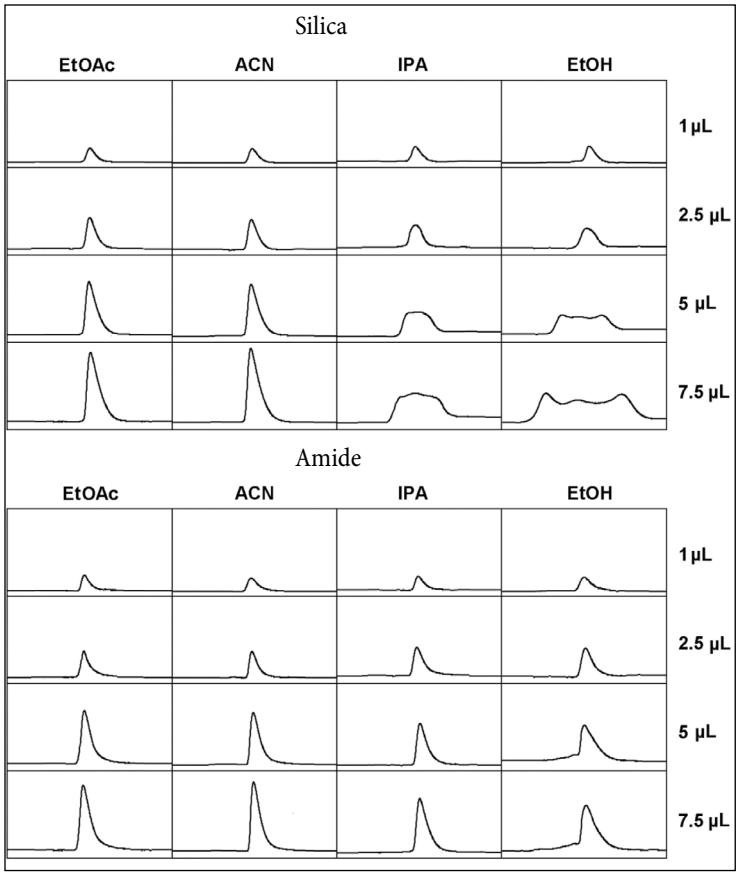

Fig. 5. The effect of the injection volume on the peak profiles of nicotine dissolved in four different solvents

phases. These data indicate that using the silica phase only nicotinic acid (dissolved in EtOAc, ACN and IPA) and benzoic acid (dissolved in EtOAc and ACN) showed no obvious peak broadening (≤10%) with increasing the injected volume. In the case of the amide phase no broadening was observed for all four analytes dissolved in EtOAc and in ACN and for nicotinic acid dissolved in IPA.

It should be noted that on the silica phase the basic analytes (melamine and nicotine) were significantly stronger affected by the injected volume compared to the acidic ones. For instance, when the injected volume increased from 1.0 to 7.5 µL, depending on the sample diluent the percent increase in peak widths was in the range 14–160% and 28–360% for melamine and nicotine, respectively. Moreover, by injecting higher volumes of nicotine dissolved in EtOH, its peak was even distorted as illustrated in Fig. 5. In contrast, under the same conditions the percent increase in peak widths for acidic compounds was significantly lower and ranged from 1.5 to 52% and from 4.4 to 22% for benzoic acid and nicotinic acid, respectively.

Influence of water content

As demonstrated above, in order to obtain adequate peak shapes in HILIC, the sample should be dissolved in an aprotic organic solvent. However, highly polar analytes often have low solubilities in pure organic solvents. It was therefore of practical importance to study the effect of water content in the sample solution on the peak efficiency of polar analytes under HILIC conditions. In the present study, increasing amounts of water in acetonitrile were tested. Figure 6 shows the peak profiles of two selected analytes (benzoic acid and nicotine) obtained on both stationary phases with the water content in the sample ranging from 0 to 60% v/v. As expected, when the content of water increased, peaks were broadened or even distorted. A similar behaviour was also observed for the other two analytes studied.

Table 5. **Percent increase in the peak width at half height as the sample volume was increased from 1.0 to 7.5 zL**

Sample solvent	Silica				Amide			
	BA	MEL	NIC	NA	BA	MEL	NIC	NA
EtAc	1.5	14	28	4.4	1.9	2.8	4.6	1.2
ACN	2.9	15	26	5.2	3.4	2.5	3.1	1.8
IPA	43	86	360	5.8	36	32	33	2.6
EtOH	52	160	Distorted peak	22	150	48	35	17

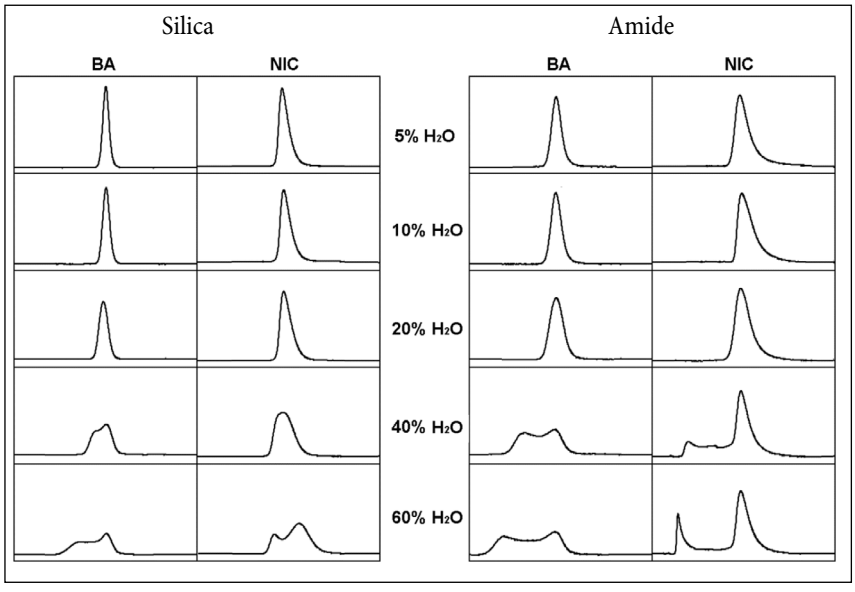

Fig. 6. The effect of water content in acetonitrile on the peak profiles of benzoic acid and nicotine

In Fig. 7 the peak widths at half height ($w_{0.5}$) are plotted as a function of water content in the sample diluent (ACN). At higher water content peak widths for benzoic acid and nicotine cannot be measured due to strongly distorted peak shapes. As can be observed, the most strongly retained nicotinic acid showed the highest resistance to the water content in the sample diluent. Acceptable peak widths of nicotinic acid were obtained when the injected sample contained ≤40% or ≤60% water for the bare silica and the amide phase, respectively. For the three less retained analytes depending on the particular analyte and the stationary phase, the critical amount of water in acetonitrile varied between 20 and 30%.

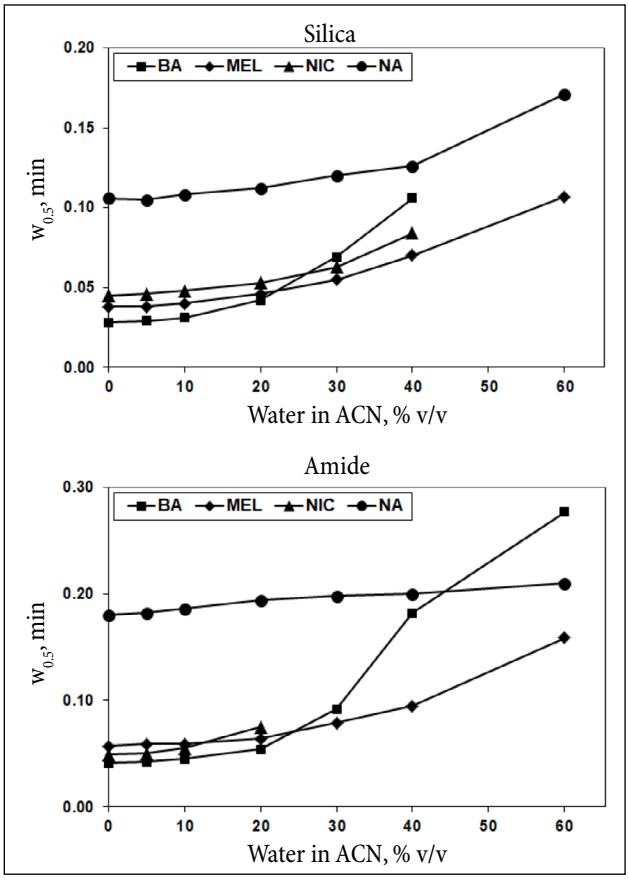

Fig. 7. The effect of water content in acetonitrile on the peak widths at half height ($w_{0.5}$) for test analytes

CONCLUSIONS

The obtained results showed that peak broadening and distortion effects noted in this study are due to a complex interaction of sample solvent properties, the injection volume and the analyte retention factor. The most significant peak shape abnormalities are caused by a mismatch between the sample solvent and the mobile phase elution strength. In general, as the solvent elution strength increases, peak broadening and distortion increases. Moreover, the aprotic character of the sample solvent was also of great importance.

Received 12 October 2017
Accepted 20 October 2017

References

1. Y. Guo, S. Gaiki, *J. Chromatogr. A*, **1074**, 71 (2005).
2. Y. Guo, S. Gaiki, *J. Chromatogr. A*, **1218**, 5920 (2011).
3. B. Buszewski, S. Noga, *Anal. Bioanal. Chem.*, **402**, 231 (2012).
4. A. J. Alpert, *J. Chromatogr.*, **499**, 177 (1990).
5. P. Hemström, K. Irgum, *J. Sep. Sci.*, **29**, 1784 (2006).
6. P. Jandera, *Anal. Chim. Acta*, **692**, 1 (2011).
7. J. W. Dolan, *LC-GC North Am.*, **22**, 26 (2004).
8. B. J. Van Middlesworth, J. G. Dorsey, *J. Chromatogr. A*, **1236**, 77 (2012).
9. J. Layne, T. Farcas, I. Rustamov, F. Ahmed, *J. Chromatogr. A*, **913**, 233 (2001).

10. S. Keunchkarian, M. Reta, L. Romero, C. Castells, *J. Chromatogr. A*, **1119**, 20 (2006).
11. J. W. Dolan, *LC-GC*, **14**, 20 (1996).
12. B. Chauve, D. Guillarme, P. Cleon, J. L. Veuthey, *J. Sep. Sci*., **33**, 752 (2010).
13. J. Ruta, S. Rudaz, D. V. McCalley, J. L. Veuthey, D. Guillarme, *J. Chromatogr. A*, **1217**, 8230 (2010).
14. *PubChem Compound Database* [https://www.ncbi.nlm.nih.gov/pccompound].
15. L. R. Snyder, J. J. Kirkland, J. W. Dolan, *Introduction to Modern Liquid Chromatography*, 3th edn., John Wiley & Sons, Inc., Hoboken, New Jersey (2010).
16. E. S. Grumbach, D. M. Wagrowski-Diehl, J. R. Mazzeo, B. Alden, P. C. Iraneta, *LC-GC North Am*., **22**, 1010 (2004).
17. C. B. Castells, R. C. Castells, *J. Chromatogr. A*, **805**, 55 (1998).

Inga Baškirova, Giedrė Rušėnaitė, Vilma Olšauskaitė, Audrius Padarauskas

MĖGINIO TIRPIKLIO ĮTAKA MAŽŲ POLINIŲ JUNGINIŲ SMAILIŲ FORMAI HIDROFILINĖS SĄVEIKOS CHROMATOGRAFIJOJE

S a n t r a u k a

Ištirta mėginio tirpiklio prigimties ir mėginio tūrio įtaka mažų polinių junginių (benzenkarboksirūgšties, melamino, nikotino ir nikotino rūgšties) smailių formai ant dviejų sorbentų (nemodifikuoto silikagelio ir amido ligandais padengto silikagelio) hidrofilinės sąveikos chromatografijos sąlygomis. Buvo tiriami šie tirpikliai: vanduo, acetonitrilas, metanolis, etanolis, izopropanolis, dimetilsulfoksidas ir etilacetatas. Ant amido sorbento tinkama smailių forma nustatyta acetonitrilo, etilacetato, etanolio ir izopropanolio tirpaluose. Silikagelio sorbente neišplitusios analičių smailės gautos tik acetonitrilo ir etilacetato tirpaluose. Geresnis amido sorbento atsparumas mėginio tirpikliui susijęs su mažesniu šios fazės hidrofiliškumu. Maksimalus nesukeliantis smailių išsiplėtimo mėginio tūris priklauso nuo mėginio tirpiklio prigimties. Didinant vandens kiekį acetonitrile, analičių smailės plėtėsi ir netgi deformavosi. Stipriausiai sulaikomai nikotino rūgščiai tinkama smailių forma gauta, kai vandens kiekis mėginyje ≤40 % silikagelio ir ≤60 % amido sorbento. Silpniau sulaikomoms analitėms, priklausomai nuo analitės ir sorbento prigimties, kritinis vandens kiekis acetonitrile svyruoja nuo 20 iki 30 %.