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A systematic investigation of the effect of sample diluent on peak shape in hydrophilic interaction liquid chromatography

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ABSTRACT

The aim of this study was to evaluate the importance of sample diluents to improve peak shapes in hydrophilic interaction liquid chromatography (HILIC), using low molecular weight (<1000 Da) analytes as well as peptides (with MW ranging between 1000 and 6000 Da) as model compounds. Various solvents were tested including water, acetonitrile, methanol, ethanol, propan-2-ol, dimethyl sulfoxide, and a number of combinations of them. For the analysis of small MW compounds, best peak shapes were obtained with sample dissolved in pure ACN but, IPA or a mixture of ACN/IPA (50:50, v/v) could represent a viable alternative in the case of solubility issues with pure ACN. For drug discovery applications, DMSO can be employed but in combination with at least 80% of ACN. For peptides analysis, acetonitrile, EtOH and IPA as sample diluents, provided similar chromatographic profiles, but pure EtOH or IPA were recommended to limit denaturation and samples solubility issues. Finally, whatever the nature of the compounds, it is recommended to add the lowest amount of water to the sample diluent, to maintain suitable peak shapes.

1. Introduction

Although RPLC remains the method of choice for the separation of various analytes, a lack of retention is often evident for the most polar compounds. While the technique certainly has earlier origins, in 1990, Alpert [1] proposed the term hydrophilic interaction liquid chromatography (HILIC), to describe an alternative method for the separation of polar analytes such as proteins, peptides, amino acids, nucleotides, and carbohydrates. HILIC is typically characterized by a hydrophilic stationary phase (such as bare silica or silica derivatized with various polar functional groups including amine, amide, cyano or diol [2]) and a mobile phase consisting of a mixture of water usually with more than 70% of organic solvent (generally ACN or MeOH).

HILIC is quite complex and some attempts have been made for better understanding mechanisms of interaction [2–5]. A multimodal retention mechanism could occur, involving (i) partitioning between a water-enriched layer at the stationary phase surface and the less polar eluent of the mobile phase, (ii) hydrogen bonding between compounds of interest with the thin water layer and/or with the silanol groups, and (iii) ion-exchange between charged analyte and the stationary phase made of bare silica or functionalized silica.

The main advantages of HILIC, summarized in the literature [2,4,6,7], included: (i) a good ability to retain polar compounds, (ii) a high organic content in the mobile phase, leading to good spraying conditions in LC–MS interface with reduced matrix effects and thus greater sensitivity [5,8–12], (iii) a lower backpressure allowing the use of higher flow rate, longer columns and smaller particle sizes [9,13–15], (iv) a separation mechanism quite diverse from RPLC which can be used simply for tuning selectivity or in the second dimension of a 2D-LC setup [8,16–21]. On the other hand, among drawbacks, we can cite (i) high consumption of expensive ACN, (ii) long column equilibration times [22,23], (iii) lack of solubility of some compounds in high proportion of organic solvent, leading to precipitation [6], (iv) peak shapes often distorted compared to RPLC [24].

In the case of method development, various parameters have to be optimized for tuning HILIC separations (in terms of selectivity and peak shapes). The influence of mobile phase composition, temperature, as well as stationary phase nature has been evaluated in detail [2,4,6,7,24–30]. However, to our knowledge, the nature of the sample diluent has never been investigated in a systematic way. In RPLC, it is well established that the sample should be dissolved in a solvent as close as possible to, or in a weaker eluent, than the mobile phase, to limit as much as possible band spreading, particularly critical for weakly retained analytes in isocratic mode [31]. In addition, it has been shown that the sample viscosity could also be a critical issue. Indeed, when the sample viscosity greatly exceeded that of the mobile phase, flow instabilities could occur which lead

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to non-uniform flow in the radial and axial directions. This phenomenon called "viscous fingering" has been reported in RPLC [32–35] and could lead to distorted peak shapes and contributed to peak broadening. According to a recent published preliminary study [14], the nature of dissolution solvent appeared to be critical for peak shape in HILIC. In the present contribution, the effects of the sample diluent on band broadening have been systematically evaluated using small compounds and peptides (with MW ranging from 1000 to 6000 Da) as model compounds. Various solvents were tested for dissolving samples including water, acetonitrile, methanol, ethanol, propan-2-ol, dimethyl sulfoxide and a mixture of them in different proportions. Finally, this work was carried out with two different HILIC materials, namely bare (hybrid) silica and silica derivatized with amide functional groups.

2. Experimental

2.1. Chemical and reagents

Water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Acetonitrile and methanol were of HPLC gradient grade from Panreac Quimica (Barcelona, Spain), ethanol and isopropanol (IPA) were from Acros organics (Morris Plains, USA) and dimethyl sulfoxide (DMSO) was from Riedel-de Haën (Seelze, Germany). Ammonium hydroxide and trifluoroacetic acid (TFA) were from Sigma–Fluka (Buchs, Switzerland). Formic acid and acetonitrile (used in MS analysis) were of ULC–MS grade and purchased from Biosolve (Valkenswaald, Netherlands).

Procainamide hydrochloride, bumetanide, lidocaine, bupropion hydrochloride, fentanyl citrate, trimipramine, esmolol hydrochloride, celiprolol, metoprolol tartrate salt, acebutolol hydrochloride, strychnine, atenolol hypoxanthine, cytosine, nicotinic acid, nadolol and acenaphthene were supplied by Sigma–Fluka, carteolol was obtained from Novopharma (Zug, Switzerland).

The model peptides used in this study were triptorelin (MW = 1312 Da), a decapeptide marketed under the brand names Decapeptyl; Diphereline and Gonapeptyl used in the treatment of hormone-responsive cancers; human insulin (MW = 5807 Da), a small protein hormone composed of 51 amino acid residues and used medically to treat some diabetic patients; arginine vasopressin and lysine vasopressin (MW = 1084 and 1056 Da respectively), two nonapeptides that differ solely by one amino acid and possess an antidiuretic and a vasopressor action, respectively. These peptides were obtained from Ferring Pharmaceuticals (Kiel, Germany), NIBSC (London, England) and Sigma–Fluka, respectively. Five other peptides namely peptides A to E with MW of 1085.2; 1598.7; 1708.8; 2180.3 and 4076.08 Da, respectively were kindly provided by a company that cannot be named due to a confidentiality agreement, the sequence of these five peptides cannot be specified.

For low molecular weight compounds, all injected samples were prepared by appropriate dilution of a 1 mg/mL stock solution of each compound. The 1 mg/mL stock solutions were prepared in MeOH for nadolol, strychnine, lidocaine, acebutolol, metoprolol, celiprolol, bupropion, bumetanide and trimipramine, in ACN for fentanyl and in ACN/water (50:50, v/v) for esmolol, atenolol and carteolol.

For peptide analysis, a stock solution at 1 mg/mL of each compound was prepared for lysine vasopressin, arginine vasopressin and peptide E in pure water, for peptide B in ACN/water (18:82, v/v) and for peptide C in ACN/water (50:50, v/v). A solution at 5 mg/mL of each compound was prepared with pure water for peptide A and D and in 0.1% TFA in water for insulin. For triptorelin, an injectable solution of 100 μg mL^{-1} (Decapeptyl, Ferring) was directly considered as stock solution.

For all compounds, final dilutions were performed with the appropriate sample diluent, to have the largest possible proportion of the desired dissolution solvent (around 95.5 and 97% in the mixture of peptides and small compounds, respectively). Thus, the quantity of solvent used for the preparation of the stock solution was negligible.

For all injections, acenaphthene was systematically added as column dead time marker and a sample blank was injected using similar chromatographic conditions. Afterwards, a blank subtraction was carried out using Empower v2.0 software to limit baseline drift at low wavelengths.

For peptides, TFA 0.1% was added to water (pH around 2) and ACN because ion-pairing between peptides and TFA leads to reduced electrostatic interactions between peptides and deprotonated silanols on stationary phases inducing better peak shapes [36]. For experiments with low molecular weight compounds, ammonium formate buffer 50 mM was prepared with formic acid and the pH was adjusted to 3.14 with ammonium hydroxide. pH values were measured in the aqueous portion of the mobile phase, but in the HILIC mode, the apparent mobile phase pH can be significantly different since the mobile phase consisted mostly of organic modifier. Rosés and co-workers described equations and factors allowing the understanding and prediction of the apparent pH for formate buffer when using acetonitrile as an organic modifier [37]. However, the authors also stated that the proposed equations were only valid for a proportion of organic modifier up to 60%, which is out of the HILIC range. In addition, the apparent pH for mobile phase containing TFA has never been evaluated with acetonitrile as organic modifier. Finally, to the best of our knowledge, the only study discussing about the variation of pH for an ACN proportion up to 90%, has been performed in absence of additives or buffers [38].

2.2. Instrumentation

Chromatographic experiments were performed using a Waters Acquity ultra performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA) that can deliver mobile phases at pressures up to 1000 bar. This instrument is equipped with a binary solvent manager with a maximum delivery flow rate of 2 mL min^{-1}, an autosampler with a 2, 5 or 10 μL loop operating in the full loop injection mode, a UV–vis programmable detector with a 500 nL flow cell set at 214 nm, and a column manager composed of a column oven set at 30 °C. Data acquisition, data handling, and instrument control were performed by Empower Software v2.0. The acquisition rate and time constant of the UV detector were fixed at 20 Hz and 50 ms, respectively. For this system, the extra column volume (V_{ext}), and the dwell volume (V_d) were experimentally measured at 13 and 100 μL, respectively (data not shown).

The analysis of a mixture of 13 small compounds was performed on a Waters Acquity UPLC system hyphenated with a Waters TQD triple quadrupole mass spectrometer fitted with a Z-spray electrospray ionization source. The TQD instrument operated at single mass resolution of m/z 0.7 FWHM, and possesses an upper mass limit of m/z 2000. The ESCi® ionization source was used in the ESI positive mode and Selected Ion Recording (SIR) was performed. Nitrogen was used as drying gas. The capillary voltage and the source extractor voltage were set at +3.5 kV and +3 V, respectively. The source temperature was maintained at 140 °C, the desolvation gas temperature and flow at 250 °C and 500 L/h, respectively, and the cone gas flow at 50 L/h. Cone voltage was set at 30 V for all compounds. Finally, dwell time and inter-channel delay were set to 20 and 5 ms, respectively, to maintain enough data points across the peaks. Data acquisition, data handling and instrument control were performed by Masslynx v4.1 Software.

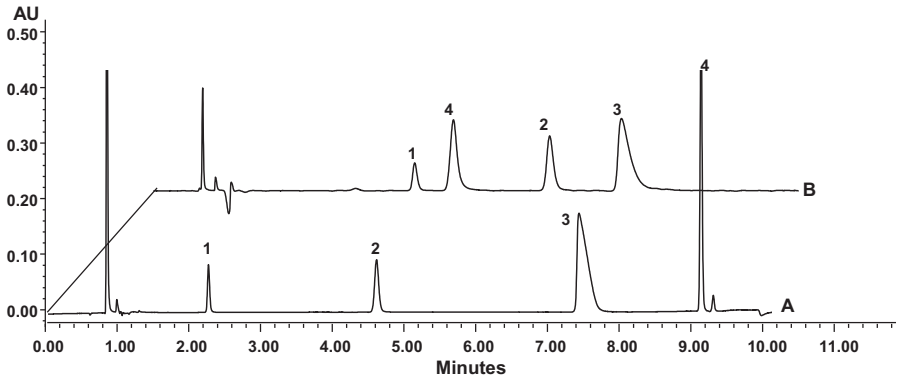


Fig. 1. Performance comparison of two HILIC columns: (A) an Acquity BEH HILIC (2.1 mm id × 150 mm, 1.7 μm) and (B) an Acquity HILIC amide (2.1 mm id × 150 mm, 1.7 μm) for the analysis of a mixture of hypoxanthine (1: 80 μg/mL), cytosine (2: 10 μg/mL), nicotinic acid (3: 30 μg/mL) and procainamide (4: 30 μg/mL) dissolved in pure ACN. Conditions: mobile phase: ammonium formate (50 mM, pH 3.14) modified with ACN, gradient profile: 95% ACN for 6 min, then 95–75% ACN in 5 min for (A) and isocratic conditions: 94% ACN for (B), flow rate of 500 μL/min, λ = 214 nm, volume injected = 5 μL, T = 30 °C.

The columns employed in this study were a bare (hybrid) silica, namely Acquity BEH HILIC column (2.1 mm id × 150 mm, 1.7 μm), an Acquity BEH HILIC amide column (2.1 mm id × 150 mm, 1.7 μm) and an Acquity BEH C18 column (2.1 mm id × 150 mm, 1.7 μm), all from Waters.

3. Results and discussion

3.1. Analysis of small molecules

One of the most important parameters to obtain suitable peak shapes in HILIC mode is the nature of the dissolution solvent, which appears more critical than in RPLC [14]. In a first instance, its influence was demonstrated for a set of model compounds. Hypoxanthine (1), cytosine (2), nicotinic acid (3) and procainamide (4) were selected (see Table 1) because of their high polarity and the fact that they could not be retained in RPLC (k values for all the compounds were comprised between 0 and 0.87 for an isocratic composition of 10:90, ACN:H_2O). Thus, their inherent properties make them good model candidates for an analysis in HILIC [14,39].

When dealing with HILIC separation, it is recommended to work with buffered eluents to reduce electrostatic interactions between charged analytes and deprotonated silanol groups at the surface of the stationary phase but also to maintain the mobile phase pH and the ionisation state of silanol groups constant. Thus, ammonium formate at pH around 3 was selected as a generic aqueous eluent, due to its buffer capacity, good solubility in high concentration of ACN and compatibility with MS detection [2].

Two different types of stationary phases, i.e. a native hybrid silica phase and a neutral amide hybrid phase were investigated. As HILIC separations are carried out with high concentrations of ACN (generally > 70%, v/v), analytes were initially dissolved in pure ACN for column performance comparison. Fig. 1 shows the chromatograms obtained for the two investigated stationary phases and the analytes elution order was 1 < 2 < 3 < 4 for the bare (hybrid) silica column, and 1 < 4 < 2 < 3 for the BEH HILIC amide support. This change could be related to the physico-chemical properties of the molecules (Table 1). Indeed, procainamide (compound 4) has a strong basic moiety (p$Ka \sim 9$) compared to cytosine, nicotinic acid or hypoxanthine which also possess a weak basic group (p$Ka \leq 4.8$). In acidic conditions, the protonated amino group could interact via electrostatic interaction with the partially deprotonated silanols of the bare (hybrid) silica stationary phase. Thus, this compound was more retained on this type of column than on the amide one which presents less underivatised silanol groups [39]. In addition, it can be noted that except for nicotinic acid (compound 3) which exhibited a pronounced tailing on both columns (USP tailing = 4.0 and 2.4 for (A) and (B) respectively), the other compounds were eluted as sharp and symmetric peaks. It can also be seen from Fig. 1, that the retention on bare (hybrid) silica columns (A) and HILIC amide (B) could be quite different for the set of model compounds. Indeed, compounds 1 and 2 were more retained on the HILIC amide while compounds 3 and 4 were less retained on this chromatographic support. Obviously, the compounds eluted during the course of the gradient were narrower (peak 4 in Fig. 1A) and for this reason, the bare (hybrid) silica column (A) was selected for the rest of this study.

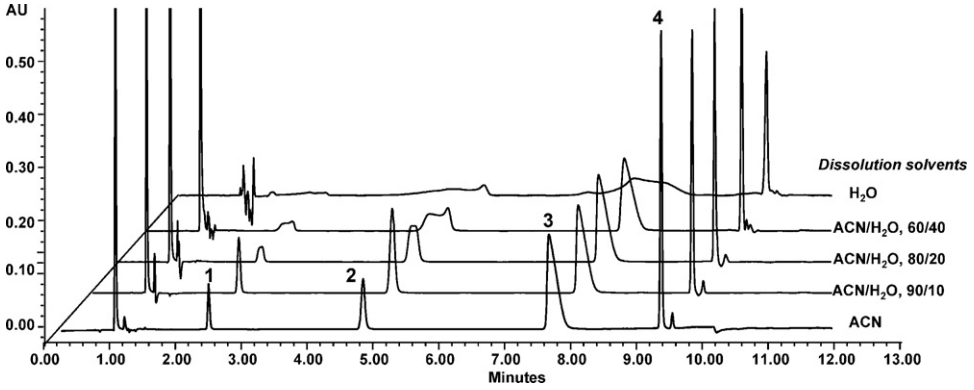
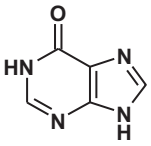
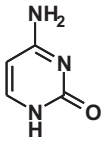
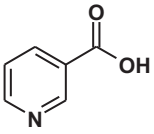
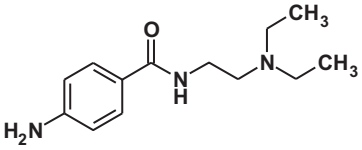


Fig. 2. Effect of the proportion of water in the dissolution solvent on the peak shape. Conditions: column Acquity BEH HILIC (2.1 mm id × 150 mm, 1.7 μm), flow rate of 500 μL/min, λ = 214 nm, volume injected = 5 μL, gradient profile: 95% ACN for 6 min, then 95–75% ACN in 5 min with T = 30 °C. Elution order: (1) hypoxanthine (80 μg/mL), (2) cytosine (10 μg/mL), (3) nicotinic acid (30 μg/mL), and (4) procainamide (30 μg/mL).

Table 1
properties of small model compounds (p*K*a, log *P* and log *D* values were calculated using Advanced Chemistry Development (ACD/Labs) Software V11.02).

Name	Formula	MW (g mol^{-1})	p*K*a	Log *P*	Log *D* (pH 3)
Hypoxanthine (1)		136.11	8.9 (acid)2.2 (basic)	−1.2	−1.31
Cytosine (2)		111.1	9.0 (acid)4.2 (basic)	−2.3	−4.13
Nicotinic acid (3)		123.11	2.2 (acid)4.8 (basic)	0.2	−1.23
Procainamide (4)		235.33	9.1 (basic)	1.1	−2.13

As ammonium formate buffer was not added to ACN, the gradient program was quite complex and consists in a simultaneous variation of ionic strength, pH and water proportion.

Finally, the stability of the bare (hybrid) silica support was tested by performing a system suitability test regularly. After around 300 injections, an acceptable decrease of less than 20% in efficiency was observed, while the peak shape, selectivity and backpressure remained satisfactory. Thus, the reported data can be considered as reliable.

3.1.1. Effect of various dissolution solvents on peak shape

3.1.1.1. Influence of water proportions. In HILIC, an important degradation of the peak shape is observed when large amounts of water are included in the sample diluent. Numerous papers can be found to illustrate the result of an inappropriate dissolution solvent [5,14,24].

In the present study, different proportions of water in ACN were tested, to define the maximum amount of water acceptable for dissolving samples, before deterioration of peak shape. Fig. 2 shows the chromatograms corresponding to an increasing proportion of water in ACN (0, 10, 20, 40 and 100% water) in the dissolution solvent and in Fig. 3 the corresponding peak widths ($w_{50\%}$) were plotted in function of the water proportion within the sample diluent. Pure ACN or 10% water in ACN generated satisfactory asymmetries and peak widths ($w_{50\%}$ values remained acceptable: $0.03 < w_{50\%} < 0.08$ for compounds 1, 2 and 4. However, as noted previously, nicotinic acid (3) exhibited a pronounced tailing ($w_{50\%}$ around 0.16) even in pure ACN. With a proportion of water between 20 and 40%, peaks eluted during the 95% ACN isocratic step (peaks 1 and 2) were distorted and splitted, while the two peaks eluted during the course of the gradient (peaks 3 and 4) remained satisfactory. Between 10 and 40% of water in ACN, a two-fold increase in $w_{50\%}$ values was observed for compounds 1 and 2 for each 10% increase, whereas $w_{50\%}$ values remain almost constant for compounds eluted in the gradient mode. Finally, with pure water, the first three peaks were strongly affected (and even disappeared) and the last peak began to tail, leading to a loss of sensitivity. No $w_{50\%}$ value could be reported for hypoxanthine (1) in pure water (Fig. 3) due to the strongly distorded peak shape (see Fig. 2).

As a rule of thumb (and similarly to RPLC), the less retained peaks (peaks 1 and 2) are always more affected by the dissolution solvent because of the difference in elution strength between mobile phase (95% ACN) and dissolution solvent. On the other hand, analytes eluted during the gradient were less affected because the proportion of buffer in the mobile phase was more important (95–75% ACN) [40].

Another remark concerns the injected volume which was adapted to the column volume (V_{inj} = 5 μL, representing about 1.4% of a column of 360 μL). Based on our own experience, for such low injected volume, a difference of 20–40% ACN between mobile phase and dissolution solvent in RPLC produces suitable peak shapes. Even if this statement is certainly not valid for every combination of stationary phase/mobile phase/solute, the behaviour in HILIC is always far more critical, as shown in Fig. 2.

Consequently, using our conditions with 95% ACN in the mobile phase, it is not recommended to dissolve analytes with more than 10% water in HILIC mode, to reach acceptable peak shapes. However, this rule can be by-passed when analytes are strongly retained (as there are less affected, as demonstrated previously) or/and when the injected volume is reduced to its minimum (significantly

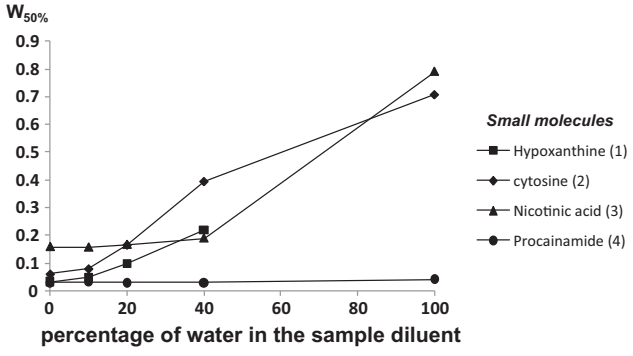


Fig. 3. Evolution of peak width at 50% ($w_{50\%}$) as a function of the water proportion in the sample diluents for the analysis of small molecules. Experimental conditions were the same as in Fig. 2. Value was not reported for hypoxanthine in pure water because of the distorted peak shape.

Table 2
Properties of various solvents used as sample diluents [43] (the dielectric constant (ε) of a solvent reflects its polarity. ε° is a measure of the degree of the adsorption interaction of the solvent with the stationary phase, it defines a solvent strength parameter based both on the characteristics of the solvent and the stationary phase. $\varepsilon^{\circ}_{(SiOH)}$ were calculated accordingly to the references [44,45] where $\varepsilon^{\circ}_{(SiOH)} = 0.77 \times \varepsilon^{\circ}_{(alumina)}$ π^* gives a good measure to the extend to which the solvent stabilizes ionic or polar species. α scale is a measure of the solvent's ability to act as a hydrogen bond donor to a solute. β scale is a measure of the solvent's ability to act as a hydrogen bond acceptor from a solute).

Solvents	ε	$\varepsilon^{\circ}_{(SiOH)calc.}$	π^*	α	β	Solvent properties
ACN	36	0.50	0.75	0.19	0.40	Aprotic polar
DMSO	47	0.48	1.0	0.00	0.76	Aprotic polar
IPA	20	0.63	0.48	0.76	0.84	Protic polar
EtOH	25	0.68	0.54	0.83	0.75	Protic polar
MeOH	33	0.73	0.60	0.93	0.66	Protic polar
H_2O	78	$\gg$1	1.1	1.17	0.47	Protic polar

less than 1% of the column volume, see Fig. 6). This issue could be particularly critical for on-line 2D-LC setup involving RPLC in the first dimension and HILIC in the second one.

When solubility of polar compounds is an issue with pure acetonitrile, samples can be dissolved in an equivolume mixture of ACN and water, ACN and MeOH or MeOH and water. Fig. 4 shows the corresponding separations of our model compounds when the injection solvent contained ACN/water (50:50, v/v), MeOH/water (50:50, v/v), and ACN/MeOH (50:50, v/v). The chromatograms were in agreement with the previous observations and the presence of an important amount of water (50%, v/v) led to considerable peak distortion. An unacceptable degradation of peaks 1, 2 and 3 was noticed, while only the most retained compound showed suitable peak shapes. When analytes were dissolved in ACN/MeOH (50:50, v/v), to limit as much as possible the presence of water, peak shapes were slightly improved but remained asymmetrical and therefore these dilution mixtures should be avoided. These results are in agreement with the study performed by Chauve et al. [14]. Indeed, the authors showed that for the analysis of a mixture of five model analytes (acenaphthene, uracil, hypoxanthine, adenine and cytosine) on a bare (hybrid) silica stationary phase, in isocratic conditions (ACN/5 mM aqueous ammonium formate pH 3 buffer, 95:5 (v/v)), better peak shapes were obtained with ACN/water (95:5, v/v) as sample diluent compared to mixtures containing a higher proportion of water, MeOH instead of ACN or an equivolume mixture of ACN/MeOH.

3.1.1.2. Effect of alternative organic solvents. Because analyte solubility is one of the major issues in HILIC, the possibility to use alternative solvents was evaluated on the bare (hybrid) silica column. Thus, the effect of various polar protic solvents (water, MeOH, EtOH and IPA) and a polar aprotic solvent (ACN), were investigated in terms of resulting peak shapes. Some of the solvents employed in the present study were previously tested as organic modifier for HILIC mobile phase [41] to (i) tune selectivity and thus improve resolution and (ii) decrease ACN consumption, particularly critical during the ACN shortage [26,42]. Table 2 summarizes properties of all the considered solvents [43]. A first remark in Fig. 5 concerns the chromatographic profile of the sample diluted in pure acetonitrile. As shown, the peak height and area observed when diluting the sample in pure acetonitrile is significantly higher than with the other sample diluents. However, the same injected quantity (concentration and volume) was considered for all these experiments. As solubility of our model compounds is not a problem in any of these solvents at this concentration, this behaviour is certainly related to the detection and should deserve some additional experiments. As shown in Fig. 5, in presence of a polar aprotic solvent (ACN) with low hydrogen bonding donor capability (α of 0.19), peaks were narrow and symmetric. For a polar protic solvent such as IPA, which also possesses quite low hydrogen bonding donor capability (α of 0.76), peaks shapes remained acceptable even if the two first peaks, eluted in isocratic conditions, were broader (N comprised between 3200 and 11,200 for IPA and between 10,500 and 28,000 for ACN) and the first one presented a non negligible fronting ($As_{10\%}$ of 0.48). Then, when hydrogen bonding donor capability became more important (α ranges between 0.83 and 1.17 for EtOH, MeOH and water) peaks were more and more distorted (see Fig. 5). The solvent polarity described by the dielectric constant, ε presented in Table 2 does not explain the difference in peak shapes between the considered dissolution solvents, while the hydrogen bonding donor capability (α) seems to be a suitable descriptor.

As a conclusion, it seems that the common dissolution solvents which are able to form hydrogen bonding (important α value) are not suitable for dissolving samples in the HILIC mode, as distorted peaks are generally observed. Indeed, because of their strong ability to form hydrogen bonding, polar protic solvents might disturb water layer at the HILIC stationary phase surface. As a consequence, peak shapes may be deteriorated. In addition, and as expected, peaks eluted during the gradient were less affected by the sample diluent composition. To sum-up, the mobile phase solvent strength increases in HILIC in the order ACN < IPA < EtOH < MeOH < H_2O as described by the ε° parameters calculated for silica adsorbent (see Table 2) [41,42,44,45]. Thus, the injection solvent merely acts as a plug of strong mobile phase which distorts the peak shape of the solutes.

According to Fig. 5, best peak shapes were obtained with ACN and IPA, although the latter produced a decrease of chromatographic performance (around 60% efficiency reduction for the peak 1 and 2 eluted in isocratic conditions). Because the sample solubility in pure ACN could be an issue, a mixture of ACN and IPA (50:50, v/v) could be used as dissolution solvent, in combination with a relatively low injected volume representing between 0.5 and 1.5% of the column volume. This solution has been experimentally achieved (see Fig. 6) and all peaks were narrow and symmetric with a 2 μL injection, representing only 0.5% of the column volume. When the injected volume increased to 5 μL (1.5% of column volume) and 10 μL (3% of column volume), peaks were broader and a loss of 50 and 70% of efficiency was observed, respectively. Therefore, during the method development, a compromise has to be found between peak width, sensitivity and solubility.

Dimethyl sulfoxide (DMSO) can be also a good candidate as it is recognized as the most powerful of any readily available organic solvents, due to its ability to dissolve a wide variety of organics substances at high concentration [46]. In drug synthesis process (e.g. combinatorial chemistry), drugs of interest are often prepared in DMSO. However, in RPLC, the injection of compounds dissolved in DMSO often results in peak distortion and insufficient retention, especially for poorly retained polar basic analytes [47]. In HILIC mode, the use of DMSO as dissolution solvent has only been scarcely investigated and only one study [5] showed poor peak shapes under HILIC conditions. However, when 25% DMSO was mixed with 75% ACN, better results are observed [5]. Accordingly, the test mixture of low molecular weight analytes was dissolved in various proportions of DMSO. Fig. 7 shows the corresponding chromatograms. Due

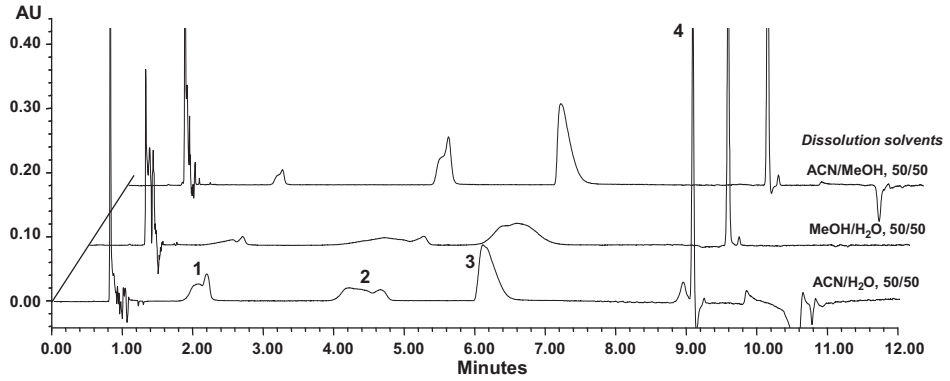


Fig. 4. Effect of the type and the proportion of organic eluent in the dissolution solvent for a mixture of (1) hypoxanthine (80 μg/mL), (2) cytosine (10 μg/mL), (3) nicotinic acid (30 μg/mL), and (4) procainamide (30 μg/mL). Conditions: column Acquity BEH HILIC (2.1 mm id × 150 mm, 1.7 μm), flow rate of 500 μL/min, λ = 214 nm, volume injected = 5 μL, gradient profile: 95% ACN for 6 min, then 95–75% ACN in 5 min with T = 30 °C.

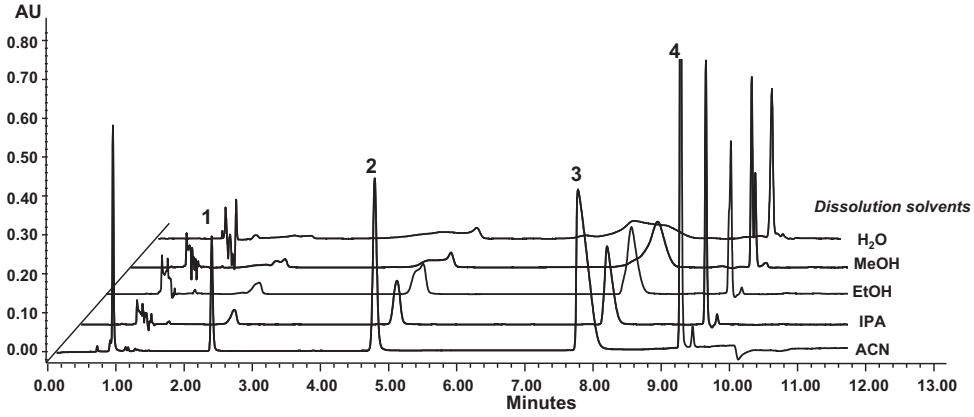


Fig. 5. Effect of different organic solvent in the dissolution solvent for the mixture: (1) hypoxanthine (80 μg/mL), (2) cytosine (10 μg/mL), (3) nicotinic acid (30 μg/mL), and (4) procainamide (30 μg/mL). Conditions: column Acquity BEH HILIC (2.1 mm id × 150 mm, 1.7 μm), flow rate of 500 μL/min, λ = 214 nm, volume injected = 5 μL, gradient profile: 95% ACN for 6 min, then 95–75% ACN in 5 min with T = 30 °C.

to a strong absorbance of DMSO at 214 nm and to avoid baseline disturbance, the first 4 min of the run was performed at 350 nm, then the wavelength was switched to 214 nm. Thus, compounds 1 with a weak retention factor could not be detected. When the proportion of DMSO in ACN increased from 0 to 20%, peaks became broader (for peak 2, eluted in isocratic conditions, N decreased from 30,000 to 21,000 to 10,000, with 0, 10 and 20% DMSO, respectively) but shapes remained acceptable. When the DMSO content increased from 50 to 100% DMSO, the two first peaks disappeared. This observation was surprising because DMSO has no hydrogen bond donor capability (α = 0.0). This result suggested that hydrogen bonding capability is not the only parameter to assess peak shapes in HILIC. However, in drug discovery [48] where DMSO is commonly used as sample dissolution solvent, HILIC can be employed as an alternative to RPLC but the compounds should be dissolved in less than 30% of DMSO in presence of ACN to obtain satisfactory results.

Finally, a valuable alternative to the water-miscible sample diluents employed throughout this study in HILIC would be the water-immiscible solvents (e.g. ethyl acetate, isopropyl acetate, methyl isobutyl ketone) that have been recently successfully employed by Loeser et al. in a recent study conducted in RPLC [49].

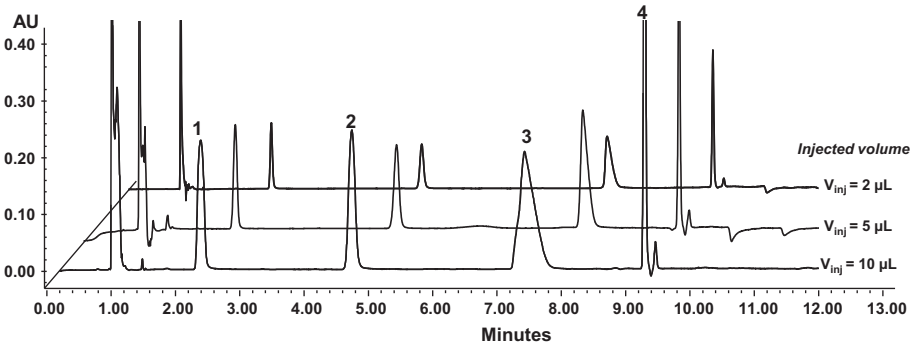


Fig. 6. Evaluation of the peak shape obtained by the use of a mixture of ACN/IPA (50:50, v/v) as dissolution solvent for the analysis of the mixture: (1) hypoxanthine (80 μg/mL), (2) cytosine (10 μg/mL), (3) nicotinic acid (30 μg/mL), and (4) procainamide (30 μg/mL) injected at various volumes (2, 5 and 10 μL). Conditions: column Acquity BEH HILIC (2.1 mm id × 150 mm, 1.7 μm), flow rate of 500 μL/min, λ = 214 nm, gradient profile: 95% ACN for 6 min, then 95–75% ACN in 5 min with T = 30 °C.

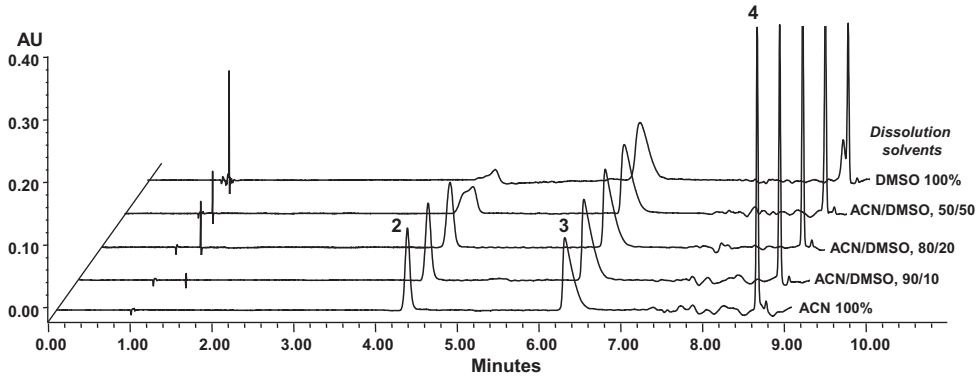


Fig. 7. Effect of the presence of different proportions of DMSO in ACN in the dissolution solvent for the analysis of the mixture: (1) hypoxanthine (80 μg/mL), (2) cytosine (10 μg/mL), (3) nicotinic acid (30 μg/mL), and (4) procainamide (30 μg/mL). Conditions: column Acquity BEH HILIC (2.1 mm id × 150 mm, 1.7 μm), flow rate of 500 μL/min, λ = 350 nm for 4 min then 214 nm, volume injected = 5 μL, gradient profile: 95% ACN for 6 min, then 95–75% ACN in 5 min with T = 30 °C.

3.1.2. Application

A separation of 13 pharmaceutical compounds, with MW ranging between 200 and 400 g/mol, was carried out using the bare (hybrid) silica stationary phase and MS as detection device. The selected analytes were diverse, including β-blockers, local anaesthetics, analgesics and antidepressors and sufficiently retained in HILIC mode (an isocratic step at 95% ACN was necessary to retain compounds 1 and 2). The whole mixture could also be analyzed by RPLC. In a first instance, the compounds of interest were dissolved in the best sample diluent, pure ACN. Fig. 8A shows the corresponding chromatogram and demonstrates the possibility to attain symmetrical peaks, possessing widths comparable to RPLC. Thus, HILIC can be considered as a complementary analytical tool to RPLC for the separation of polar compounds but also as an alternative to RPLC for the analysis of less polar compounds (to improve sensitivity with MS detection or tune selectivity). A mixture of ACN/IPA (50/50, v/v) was also evaluated as sample diluent to resolve a potential solubility issue of polar analytes in pure ACN. As reported in Fig. 8B, except peaks 2 and 3 which were slightly broader compared to pure ACN, peak shapes were fully comparable in the two tested conditions.

Thus, for the analysis of low molecular weight analytes in HILIC, it is recommended to dissolve the compounds in pure ACN or with ACN containing up to 50% IPA (v/v) as an alternative.

3.2. Analysis of peptides

Nowadays peptides are emerging as an important class of therapeutic agents in the pharmaceutical field. Thus, there is a need to develop efficient methods for assessing the quality of peptides produced by biotechnological procedures. In addition, peptides have also received attention in proteomics and peptide mapping which

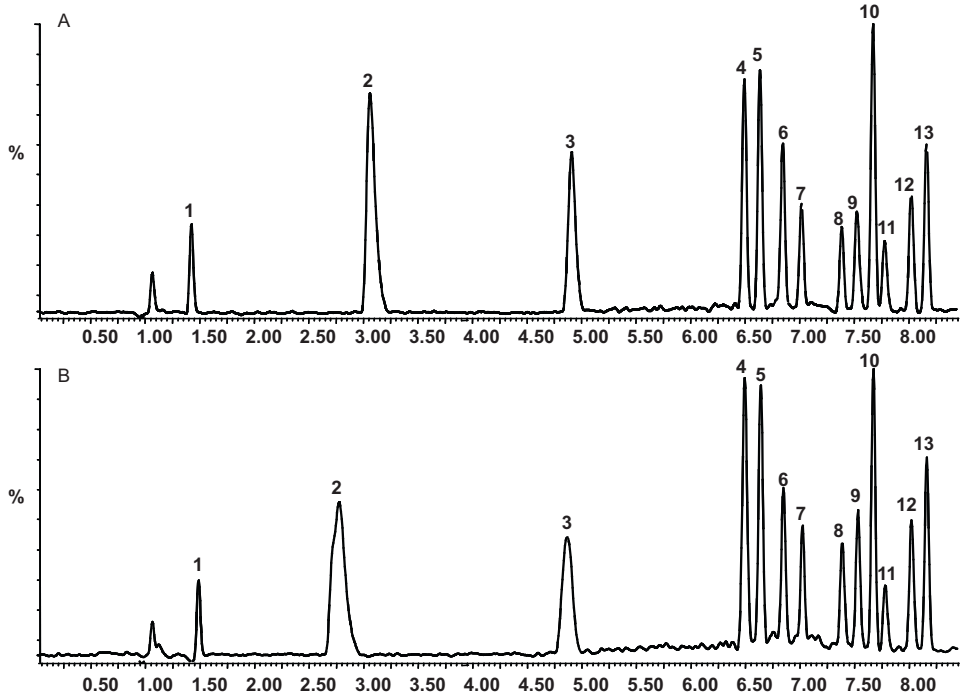


Fig. 8. Separation of 13 pharmaceutical compounds, dissolved in a large amount of (A) ACN and (B) ACN/IPA (50:50, v/v), on the column Acquity BEH HILIC (2.1 mm id x 150 mm, 1.7 μm) by UPLC–MS. Conditions: mobile phase: ammonium formate (20 mM, pH 3) modified with ACN, gradient profile: 95% ACN for 3 min, then 95–65% ACN in 12 min, flow rate of 400 μL/min, λ = 214 nm, volume injected = 2 μL, T = 30 °C. Elution order: (1) bumetanide (1 μg/mL), (2) lidocaine (0.05 μg/mL), (3) bupropion (0.4 μg/mL), (4) fentanyl (0.05 μg/mL), (5) trimipramine (0.05 μg/mL), (6) esmolol (0.05 μg/mL), (7) metoprolol (0.05 μg/mL), (8) acebutolol (0.05 μg/mL), (9) celiprolol (0.05 μg/mL), (10) carteolol (0.05 μg/mL), (11) nadolol (0.1 μg/mL), (12) strychnine (0.1 μg/mL), (13) atenolol (0.05 μg/mL).

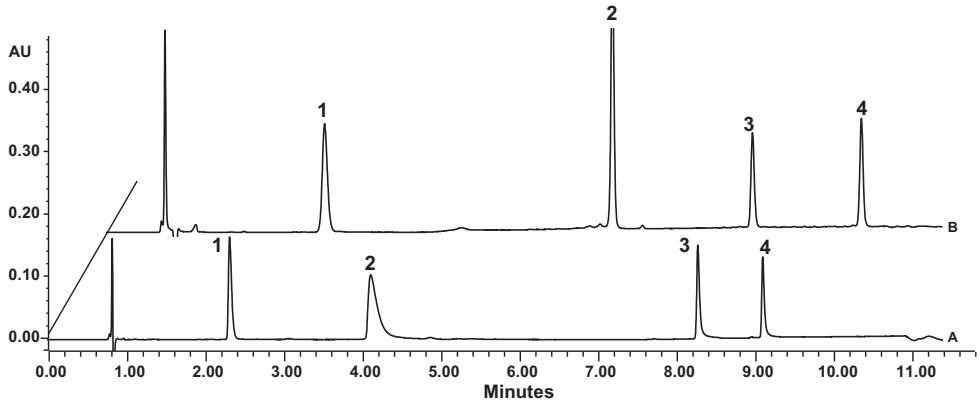


Fig. 9. Performance comparison of two HILIC columns: (A) an column Acquity BEH HILIC (2.1 mm id × 150 mm, 1.7 μm) and (B) an Acquity HILIC amide (2.1 mm id × 150 mm, 1.7 μm) for the analysis of a mixture of peptides: peptide A (15 μg/mL), peptide C (25 μg/mL), peptide D (40 μg/mL) and insulin (45 μg/mL) dissolved in large amount of ACN (see experimental section). Conditions: mobile phase: 0.1% TFA in water modified with 0.1% TFA in ACN, gradient profile: 94% ACN for 5 min, then 94 to 75% ACN in 6 min for (A) and gradient profile: 90% ACN for 3 min, then 90 to 62% ACN in 9 min for (B), flow rate of 500 μL/min, λ = 214 nm, volume injected = 5 μL, T = 30 °C.

is generally performed by RPLC followed by MS detection. For analyzing peptides, HILIC appears as a valuable strategy as it provides a very high degree of orthogonality in comparison with RPLC (69%, as demonstrated elsewhere [16]), which can be attractive for tuning selectivity.

Again, the sample diluent could greatly impact on peak shape and solubility, and it was studied systematically because peptides differ strongly from low molecular weight compound. Thus, previously drawn conclusions cannot be directly extended since peptides possess higher molecular weight and more importantly, present multiple charges. Thus, their chromatographic behaviour in HILIC, based on hydrogen bonding and electrostatic interactions, could be strongly different.

For the present study, a mixture of peptides A, C, D and human insulin was used.

For peptide analysis, the use of 0.1% TFA in ACN and water was considered due to the relatively poor resolution of peptides with formic acid. The performance of the two HILIC columns: Acquity BEH HILIC and Acquity HILIC amide was also compared using ACN as dissolution solvent. Fig. 9 shows the corresponding chromatograms. The analysis performed on the bare (hybrid) silica (Fig. 9A) showed a significant peak tailing (i.e. USP tailing comprised between 1.5 and 2.8). As peptides are polycationic compounds, they could interact with negatively charged silanols of the stationary phase (even if the number of the latter should be small at pH 2), although the presence of TFA in the mobile phase is known to reduce electrostatic interactions between peptides and residual silanol groups by ion-pairing effects at the surface of silica particles [50,51]. With the neutral HILIC amide column (Fig. 9B), all peaks were symmetrical (i.e. USP tailing around 1.1) since, peptides do not interact via secondary interactions. From these observations, the HILIC amide support was found more adapted for peptides analysis, and selected for further experiments.

To ensure the proper operation of the HILIC amide column, a system suitability test was regularly performed and during the course of 300 injections required for this study, a decrease of about 30% in efficiency was observed, while peak shapes, selectivity and backpressure remained satisfactory. This reduction of efficiency is quite important for such a limited number of injections but it is worth mentioning that the analytical conditions employed throughout this study were very different, in terms of injected sample and mobile phase conditions. In addition, this variability of 30% is low in comparison with the variation of efficiency related to changes in sample diluent and injected volumes.

3.2.1. Effect of various dissolution solvents on peak shape

3.2.1.1. Influence of water proportions. For peptide analysis, the maximum amount of water which can be used without peak distortion was estimated, using a comparable procedure to that previously described. Fig. 10 shows that, in the tested conditions where the initial mobile phase contains 90% ACN, again up to 10% of water can be added to the sample diluent without affecting peak width, asymmetry and resolution, except peak 1 which was slightly broader (a 50% increased in $w_{50\%}$ was observed). In any case, when

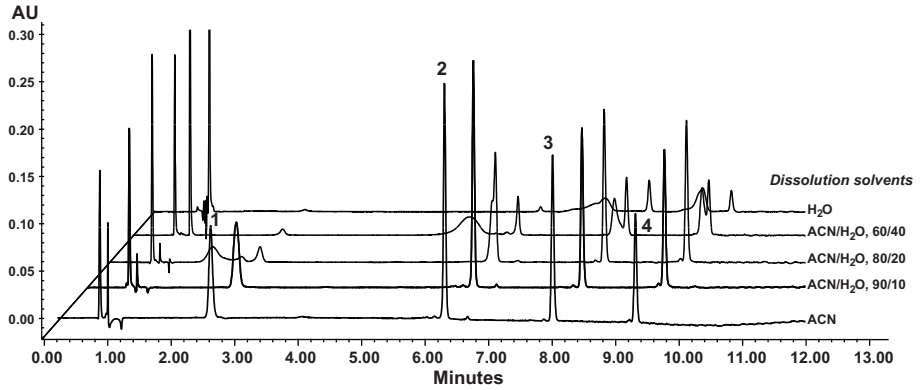


Fig. 10. Effect of the proportion of water in the dissolution solvent on the peak shape. Conditions: column Acquity HILIC amide (2.1 mm id × 150 mm, 1.7 μm), flow rate of 500 μL/min, λ = 214 nm, volume injected = 5 μL, gradient profile: 90% ACN for 3 min, then 90–62% ACN in 9 min with T = 30 °C. Elution order: (1) peptide A (15 μg/mL), (2) peptide C (25 μg/mL), (3) peptide D (40 μg/mL), and (4) insulin (45 μg/mL).

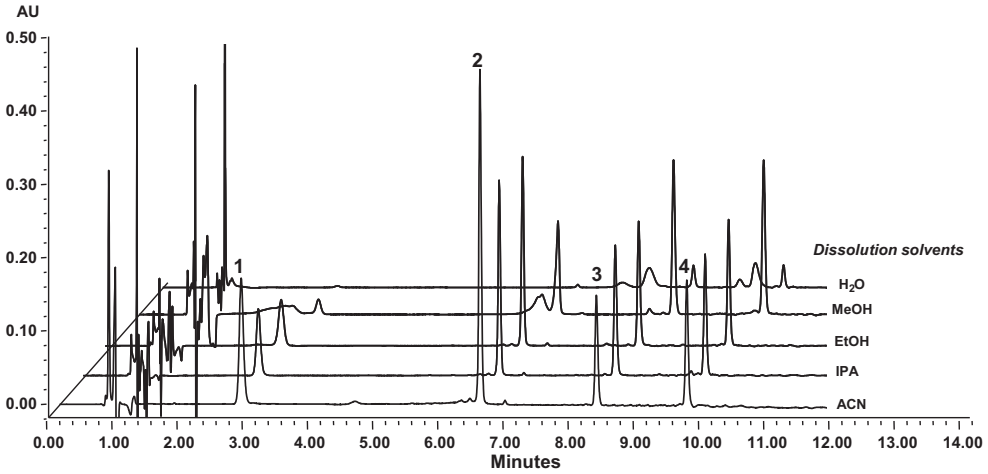


Fig. 11. Effect of different organic solvents in the dissolution solvent for the mixture: (1) peptide A (15 μg/mL), (2) peptide C (25 μg/mL), (3) peptide D (40 μg/mL), and (4) insulin (45 μg/mL). Conditions: column Acquity HILIC amide (2.1 mm id × 150 mm, 1.7 μm), flow rate of 500 μL/min, λ = 214 nm, volume injected = 5 μL, gradient profile: 90% ACN for 3 min, then 90–62% ACN in 9 min with T = 30 °C.

the proportion of water increased, peaks were broadened, split and even disappeared for the highest proportions of water. Thus, the evolution of the corresponding peak widths ($w_{50\%}$) as a function of the water proportion in the sample diluents could not be plotted.

For a proportion of water of 20%, only the two first peaks (eluted during the isocratic step) were affected, while for a larger amount of water, all peaks became distorted. To conclude, an amount of water should be added to the sample diluent only in case of solubility issue and should be always set to a minimum value.

3.2.1.2. Effect of alternative organic solvents. As previously demonstrated, an alternative strategy to limit solubility issues is to dissolve the sample in another organic solvent. Therefore, the impact of polar protic solvents (MeOH, EtOH, and IPA) and polar aprotic solvent (ACN) as dissolution solvents was also investigated for peptides analysis.

Fig. 11 shows that peaks of our four model peptides remained perfectly symmetric and narrow with ACN, IPA, and EtOH. However, when the solvent had a higher capacity to form hydrogen bonding (i.e. α > 0.9 for MeOH and water, see Table 2) peaks were distorted. In presence of MeOH, only peaks eluted during the isocratic step at 95% ACN were affected while with pure water (and as previously discussed), the four peaks almost disappeared. Then, ACN, IPA and EtOH could be potentially used as dissolution solvent for our sepa-

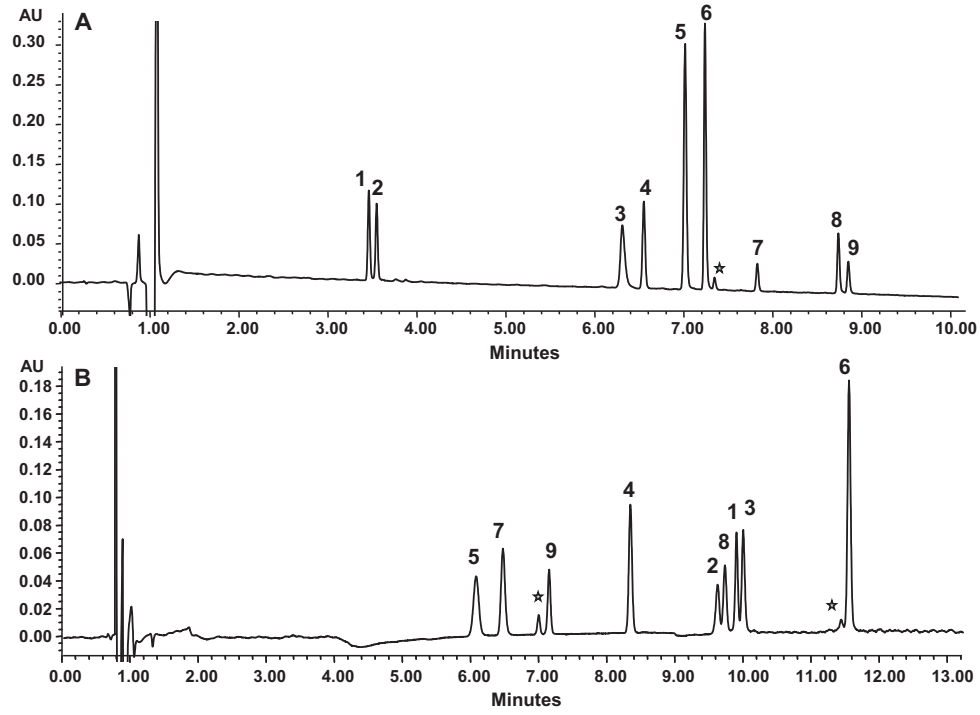


Fig. 12. Comparison of the chromatographic performance obtained by (A) RPLC *vs.* (B) HILIC for the analysis of a mixture of 9 peptides: (1) lysine vasopressin (20 μg/mL), (2) arginine vasopressin (12 μg/mL), (3) peptide D (20 μg/mL), (4) triptorelin (5 μg/mL), (5) peptide A (20 μg/mL), (6) insulin (60 μg/mL), (7) peptide B (6 μg/mL), (8) peptide E (25 μg/mL), (9) peptide C (6 μg/mL) dissolved in water for (A) and in IPA for (B); the star (*) designates an impurity present in synthetic peptides. Conditions: (A) Column Acquity BEH C18 (2.1 mm id × 150 mm, 1.7 μm), flow rate of 400 μL/min, λ = 214 nm, volume injected = 5 μL, gradient profile: 10–90% ACN in 20 min with T = 30 °C, (B) Column Acquity HILIC amide (2.1 mm id × 150 mm, 1.7 μm), flow rate of 500 μL/min, λ = 214 nm, volume injected = 5 μL, gradient profile: 90% ACN for 3 min, then 90–62% ACN in 9 min with T = 30 °C.

ration of peptides, without affecting peak shapes. For our examples, sample diluent seems to be less critical for peptides than for low molecular weight compounds analysis (see Section 3.1.1.2), where only ACN or a mixture of ACN/IPA (50/50, v/v) provided symmetric and narrow peaks. However, it is important to note that two different HILIC supports were employed (HILIC BEH for small molecules and HILIC BEH amide for peptides), and the gradient profile was different. Indeed for peptide analysis, the proportion of water in the mobile phase (gradient from 90 to 62% ACN in 9 min) was higher than for small molecular weight compounds (gradient from 95 to 75% ACN in 5 min). Thus, the use of a stronger injection solvent than ACN is less critical, as the mobile phase strength (i.e. water concentration) is greater in this example at the point of elution of the analytes.

From our point of view, the choice between ACN, IPA and EtOH should be based on both solubility and stability of the investigated peptides. The stability of our four model peptides in ACN, EtOH and IPA was evaluated after storage at room temperature for 11 days. As expected, peptides were less stable in ACN (average decrease of 40% in peak height) than in EtOH or IPA (no change in peak height after 11 days). This observation confirmed that ACN could denature peptides. For these reasons, IPA (or in some cases EtOH) is certainly the best sample diluent for peptide analysis in the HILIC mode. It is however important to note that the time of contact between the sample and the mobile phase is very short compared with the time of contact between the dissolution solvent and the sample. Thus, this explained why there is no problem to study peptides with ACN as mobile phase while sample diluent can be critical.

3.2.2. Application

A separation of 9 model peptides was performed in HILIC (Fig. 12B) using IPA as sample diluent and an isocratic step at 90% ACN for 3 min following by a gradient from 90 to 62% in 9 min. As shown, all compounds were baseline separated and peaks were narrow and symmetric. This mixture was also injected in RPLC (Fig. 12A) with a gradient from 10 to 90% ACN in 20 min, to compare the performance of these two separation techniques. Again, similar chromatographic profiles were obtained in both RPLC and HILIC, showing that the HILIC mode can be a good alternative to RPLC for the separation of peptides.

Indeed, a very different retention mechanism was demonstrated between the two separations (the correlation coefficient r was equal to −0.35 when plotting HILIC retention times vs. RPLC retention times). For example, peptides 8 and 9 were weakly separated by RPLC but well resolved in HILIC with a reversed elution order. On the other hand, peptides 2 and 8 were much better separated in RPLC than in HILIC. Finally, peptides 1 and 2, which differed only by one amino acid in the sequence, were baseline resolved with both separation mechanisms.

4. Conclusion

The effect of dissolution solvent to improve peak shapes in HILIC was investigated, in a systematic way, through the analysis of low molecular weight pharmaceutical compounds (with MW < 1000 Da) and peptides (with MW ranging between 1000 and 6000 Da).

The effect of various polar protic solvents, including water, MeOH, EtOH, and IPA, and polar aprotic solvents such as ACN, and DMSO, as sample diluents, was initially evaluated with small model analytes. It appeared that ACN remains the first choice for dissolution solvent in HILIC for small compounds analysis. But, a mixture of ACN/IPA (50:50, v/v) could be a suitable sample diluent in case of solubility issues. Based on our results, when the initial concentration of ACN is high (e.g. 95% ACN), a proportion of water greater than 10% in the sample diluent should be avoided to maintain sharp peaks.

Conclusions drawn for peptides analysis were almost similar and water should be avoided as much as possible. For the dissolution of peptide mixtures, ACN, IPA, and maybe EtOH provided similar chromatographic profiles (if the peptides are not eluted with a high proportion of ACN). Thus, IPA (or EtOH) was recommended to limit the denaturation of relatively large biomolecules sometimes observed with ACN.

It is finally worth mentioning that these conclusions have to be considered with caution as this study considered only two types of HILIC stationary phases and a restricted number of analytes. Thus, the present study provides a good starting point for HILIC method development but conditions might not be optimal for other analytes and HILIC supports.

In this work, we also demonstrated that similar chromatographic performance could be obtained in both RPLC and HILIC for small molecules and peptides, with a suitable sample diluent. Thus, HILIC can be a good alternative to RPLC, as it present a very different retention mechanism particularly useful in an on-line 2D-LC set-up, providing that HILIC, more prone to sample diluent effects, should be in the first dimension [18,19,21].

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