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Abstract

In this study, the impact of introducing water in the sample solvent upon the injection in SFC is investigated. Adsorption of water on the stationary phase was indicated. Using a set of ten neutral test compounds and four ionizable test compounds, spread all along the co-solvent gradient, several parameters were scrutinized (i.e. water content in the sample diluent, nature of the sample diluent, nature of the co-solvent) in regards to peak broadening. From this systematic investigation, the competition for adsorption on the stationary phase between the analytes and the water molecules contained in the diluent was highlighted. The chromatographic peaks of neutral molecules eluting before water molecules were compressed and the ones eluting after were broadened. While the extent of this phenomenon was related to the peak position for neutral molecules, it was not observed on acidic molecules.

Keywords

Supercritical fluid chromatography; sample diluent; injection; peak broadening

1. Introduction

In analytical SFC, the injection is performed after the co-solvent and the CO_2 are mixed at initial proportions. Hence the injection plug is located in a zone usually rich in CO_2 and a viscosity mismatch is frequent. Moreover, the nature of the sample diluent is of major importance on the peak shape. The selection of the sample diluent is hence of great concern. For polar SFC stationary phases, aprotic and low viscosity solvents, such as MtBE, are recommended to avoid the formation of viscous fingering as well as any strong solvent effects [1].

However, for the past few years, the application area of SFC is growing interest towards more polar solutes which may experience solubility issues in aprotic solvents. The use of polar co-solvents in SFC eases the analysis of polar metabolites, sugars or peptides [2], for which water is usually the best solubilizing diluent. Another interesting development for SFC is its implementation as a second dimension in a two-dimensional chromatographic separation. This combination opens new opportunities, thanks to the large orthogonality it provides [3, 4], while it may impose the transfer of hydro-organic fractions into the SFC dimension.

The presence of water and eventually MeOH is known to be highly detrimental to SFC peak shapes, as these solvents both present elevated viscosity and polarity, contrasting with the initial CO_2-rich mobile phase. So far, solutions consisted in injecting very low sample volumes. But Enmark et al.

highlighted that deformations can even happen at very low injection volume, starting at 0.3 % column volume [5]. Using specific flow-through needle injectors that increment the sample plug with a feed solvent has shown recently to be able to reduce the impact of MeOH diluent on the separation [6], but water as a diluent was not investigated. On the other hand, the situation where SFC is online with an LC instrument is even more demanding. In an online LC x SFC configuration, loops of large volume need to be used to allow sufficient time for the ^{2}D separation to be conducted. Moreover, the loops need to be fully filled to avoid residual air gaps, that may result from the decompression of the ^{2}D mobile phase in the loop, to be injected [7]. This leads to the injection of a large amount of ^{1}D mobile phase into the ^{2}D SFC dimension. Previous online hyphenation of LC with SFC avoided the water injection issue by removing the LC eluent through packed loop interfaces [8-10], or by injecting very low volumes into the SFC column [7]. While the first solution is highly effective, the risk of carry-over exists. On the other hand, the transfer of low volume is quite demanding in online two-dimensional separations, imposing a drastic reduction of the ^{1}D flow-rate. A better understanding of water impact upon injection in SFC is hence required.

Lastly, one can notice that studies focusing on injection effects of water-containing diluents in SFC have been performed under isocratic mode so far [5, 7, 11]. However, gradient elution is known to favor robustness in SFC [12] and is increasingly applied to separate analytes covering a wider range of polarity [13].

The goal of this experimental study was to conduct a systematic investigation of the influence of several parameters on band broadening when injecting samples dissolved in a water-containing diluent. These parameters included the position of the analyte peak, the water content in the injection solvent as well as the nature of the organic solvent injected along. The nature of the co-solvent and the presence of additives in this co-solvent were also investigated.

2. Materials and methods

2.1 Chemicals, reagents and columns

All standards had a purity exceeding 95%. The following standards were obtained from Sigma Aldrich (Germany): eugenol, 2,4,6-trimethylphenol, 1-indanol, apocynin, o-cresol, m-cresol, phenol, 4-hydroxybenzyl alcohol, naringenin, acrylic acid, imipramine hydrochloride and propranolol hydrochloride. Arbutin, trans-cinnamic acid and ferulic acid were obtained from Fluka (UK), Sigma Acros Organic (USA) and Sarsyntex (France), respectively.

Methanol (MeOH) (≥ 99.8 %), ethanol (> 98 %) and formic acid (> 98 %) were purchased from Fisher Scientific (UK), acetonitrile (ACN) (≥ 99.9 %) was purchased from Honeywell (Germany), methyl tert-butyl ether (MtBE) (≥ 99.9 %) was purchased from Acros Organics (USA).

Ultrapure water was delivered by PURELAB Classic system from Elga (UK) (18.2 MΩ-cm). Ammonium hydroxide solution was ACS reagent (28.0-30.0 % NH_3 basis) purchased from Sigma-Aldrich (Germany). Pressurized carbon dioxyde (CO_2) (N45, ≥ 99,995 %) was purchased from Air Liquide (France).

Three columns were assessed, Torus DEA and Torus Diol with dimensions 100 x 3.0 mm, 1.7 µm and BEH HILIC with dimensions 100 x 2.1 mm; 1.7 µm, all from Waters (Milford, USA).

2.2 SFC instrument

All experiments were performed on an Agilent 1260 Infinity II SFC (Agilent, USA) equipped with a SFC-binary pump (G4782A), an oven column 1260 MCT (G7116A), a detector 1260 DAD WR (G7115A) equiped with a SFC flow cell (400 bars, 2 µL, 3 mm path length) and a 1260 SFC control module (G4301A). To mimic the full loop injection that may occur during online LC x SFC, the commercial flow-through needle (FTN) injector was reverted to a fixed-loop model. The instrument was modified to accommodate a SFC-Autosampler (G4303A) with a fixed injection loop of either 5 µL, 10 µL or 20 µL volume. The instrumental variance due to extra column was estimated at 37 µL² (liquid conditions) and at 12 µL² (for 5 % co-solvent). The dwell volume, excluding loop volume, was 780 µL.

2.3 Sample preparation

A stock solution of each standard was prepared at 20 mg/mL or 20 µL/mL in MeOH. Using the stock solutions, a mixture containing several compounds at a final concentration between 0.05 to 0.2 mg/mL (according to their UV response) in either MtBE, or ACN/water with various ratio, or MeOH /water with various ratio, except for imipramine prepared at 1 mg/mL.

2.4 Chromatographic conditions

Samples were injected with an injection volume of 5 µL, 10 µL or 20 µL (full loop with overfill factor x2). The flow rate was 1.4 mL/min, so that the instrumental pressure remains below 400 bars during gradient. The initial composition of the mobile phase was 95 % CO_2 / 5 % organic co-solvent (v/v), with a linear gradient up to a final composition of 50 % co-solvent, with a gradient time of 5.3 min (normalized gradient slope of 2 %). Then the column returned to the initial composition in 0.3 min (corresponding to one void volume) and was reconditioned with initial conditions for 2 min. Back-pressure was maintained constant at 140 bar. The column oven temperature was set at 40 °C ± 0.8 °C. UV detection was set at 220 nm (bandwidth 4 nm, frequency 40 Hz) unless stated otherwise. The instrument was controlled by OpenLab CDS ChemStation C01.08 (Agilent). Ammonium hydroxide (20 mM) was added to the co-solvent to avoid peak tailing when analysing basic compounds [14].

2.5 Selection of test compounds and column.

While most studies on injection effect focus on poorly retained analytes, it seemed important to select compounds across the whole elution range while limiting the number of variables. A set of 10 neutral test compounds, with log P ranging from -0.6 to 2.2 was investigated, selected among lignin-derived compounds. Two smaller sets containing three acidic compounds and two basic compounds completed the study (Table 1).

Diethylamine column (DEA) was selected as providing the largest elution range and resolution for the selected test compounds. Conditions of pressure and temperature in gradient SFC have been selected to be representative of the conditions the most used in literature, while offering the largest retention range [15, 16].

A non-linear density increase and a pressure gradient along the run at fixed BPR pressure (here 140 bars) results in a dependency of retention behavior not only to gradient conditions (initial composition,

slope) but also to the operating conditions [17, 18]. For any rigorous method transfer of a linear gradient, solute retention should be reported via composition at elution expressed as co-solvent molarity [19, 20]. Nonetheless, for easiness of instrumental setup, the apparent composition at elution (Eq. 1), expressed in volumetric fraction of co-solvent was used throughout this study. This straightforward comparison of elution of analytes is possible, as all experiments were performed on a single instrumentation with highly repeatable pressure profile throughout the runs.

The composition at elution, Ce, was given by:

$$C_e = C_i + \frac{C_f - C_i}{t_g} \times (t_R - t_0 - t_D) \qquad (1)$$

with t_R the compound retention time and t_D the instrument dwell time.

C_i and C_f are the initial and final eluent compositions expressed as volumetric fractions, t_g the gradient time and t_0 the column dead time. The dwell time was determined by performing a triplicate set of gradient profiles without column, with a gradient composition ranging from 60% to 85 % co-solvent. A larger compositional range led to non-linear absorbance profile.

3. Results and discussion

This study has been carried out using generic SFC conditions with a gradient elution from 5 % to 50 % co-solvent and a set of neutral, acidic and basic test compounds.

Methyl tert-butyl ether (MtBE) is considered as a very weak solvent and the variance generated by the injection in this solvent is considered close to the one generated by heptane, while allowing compounds of a wide range of polarity to be soluble [1]. Hence it was considered through this study as the reference injection solvent. Figure 1 shows the reference chromatogram, with MeOH as co-solvent, when injecting the test compounds diluted in 5 µL MtBE. The retention times and hence the apparent composition at elution of the test compounds are reported in Table 1. Because of the large dwell volume (1.8 times the column volume), three compounds eluted on the resulting isocratic step, at the initial composition of 5 % MeOH, with retention factor ranging from 0.8 to 1.3. These compounds had a smaller column dispersion contribution and hence were expected to be highly influenced by injection effects, besides instrumental variance. Then the next seven neutral test compounds eluted in the range of 6 % to 35 % MeOH. Retention times were repeatable with intermediate precision (5 days) reaching 2.0 % RSD for the low retained test compounds and 1.3 % RSD for the test compounds eluting during the gradient.

Because the viscosity and pressure drop evolved across the gradient, molecular diffusion and hence the column efficiency were not constant through the separation. Efficiency measurements were performed with compounds exhibiting k values around 3, using isocratic composition of either 5 % MeOH or 35 % MeOH and ranged from 17 000 to 9 000 plates, respectively. Hence the peak variance, resulting from the sum of column variance, instrumental variance and injection variance could not be compared from one test compound to another. The individual peak variances σ_{MtBE}^2 were calculated from the reference chromatogram (Figure 1) and were in the range of 300-500 µL² (RSD < 10 %) for all test compounds, showing that the instrumental variance (≤ 37 µL²) was negligible under these conditions.

3.1 Adsorption of the injection solvent

Several SFC studies demonstrated that common polar solvents can exhibit retention on polar stationary phases [21, 22]. Detecting at 200 nm so that the MeOH co-solvent exhibited a significant absorbance, it was possible to follow the system peaks due to the elution of solvent molecules.

According to the displacement method [23, 24], the injection of a retained solvent produces two zones: the elution zone containing the injected molecules, called “tracer peak” that reflects the retention of the injected molecules, and the displaced elution zone called the “perturbation peak”. The injection of pure MtBE and pure ACN conducted to a single large negative perturbation peak around 0.6 min, while the injection of pure MeOH led to a right angled-triangular perturbation peak, suggesting that MeOH molecules are adsorbed on the stationary phase (Figure 2a). This MeOH adsorption on SFC stationary phases has already been evidenced [22]. The injection of pure ACN provided the exact same elution profile as the injection of MtBE, suggesting that ACN is not retained on the stationary phase. When injecting ACN/water mixtures (Figure 2b), the size and shape of this perturbation peak were not significantly affected by the amount of water. When injecting MeOH/water mixtures (Figure 2c), the injected MeOH molecules eluted in the perturbation zone. Increasing the amount of injected MeOH increased the level of UV absorbance in this zone. This suggests that the co-solvent deficiency generated by the injection of ACN/water or MeOH/water migrates at the speed of the MeOH co-solvent, as expected [24].

By injecting increasing amounts of water, it was also possible to observe a negative peak corresponding to the tracer peak of water, eluting later in the gradient. Figure 2d shows a right angled-triangular shape peak, which area increased with the injected amount of water. Using first-order moment [25], the retention time of this skewed peak was deduced at 2.00 min corresponding to a composition at elution of 13.7 % MeOH co-solvent, proving the large retention of water molecules on DEA stationary phase. Injecting water/ACN mixtures provided a tracer peak with the same retention time and shape, but slightly lower intensity. It is to be noted that during the course of these experiments using 5 µL sample volume, no difference could be observed on the pressure trace that was highly repeatable whatever the diluent.

Hence, when injecting samples in hydro-organic phases, both water and methanol molecules from the diluent competed with the analytes for the access to the stationary phase, while ACN molecules had no stationary phase interaction. Hence ACN/water diluents were expected to be less prone to generate injection effects than MeOH/water diluents.

3.2 Influence of the diluent composition on peak broadening

Studies on the effect of injection diluents on chromatographic performances have been performed by monitoring column efficiency [11, 22] or peak shapes [1]. However, the first is only applicable on isocratic separation while the latter may not properly reflect focusing/broadening phenomenon. As mentioned before [7], a slight retention shift occurred when increasing the water content in the diluent.

The contribution of the composition of the injection solvent to the process of peak broadening can be monitored as the relative variation of peak variance as compared to a similar injection with the sample diluted in MtBE. To more quantitatively describe the effect of the injection solvent on band broadening, the variance generated by the hydro-organic diluent $\sigma^2_{diluent}$ was normalized in regards to the observed variance of the same peak when using MtBE as injection solvent σ^2_{MtBE} .

Figure 3 exhibits the influence of water content in the diluent on the peak broadening. The first observation was that injecting in pure ACN diluent (grey marks) resulted in a larger peak variance for early eluting peaks, as compared to MtBE diluent and as discussed in section 3.1, and had no effect on late-eluting peaks, as expected from a strong solvent effect. On the contrary, when the diluent contained water, the first eluting peaks, with composition at elution lower than 10 %, exhibited a clear compression, i.e. their variance when injected in hydro-organic solvent was lower than when injected in MtBE. For these compounds, the presence of water in the diluent had a clear beneficial effect. Compounds eluting later than the water plug, i.e. with composition at elution higher than 15 % MeOH, encountered a broadening effect. These observations are typical from displacement and tag-along effects when a column is overloaded, here with water molecules. This effect is well documented in preparative liquid chromatography, where the retention of major solutes affects the peak shape of minor solutes [26].

In analytical SFC, Redei et al. [22] demonstrated that the retention of methanol diluent on an alkylamine column using neat CO_2 as mobile phase conducted to a focusing effect on low retained alkylbenzenes, a broadening effect on alkylbenzenes that were more retained than methanol and that alkylbenzenes that were greatly retained were unaffected by the presence of the diluent. Here we confirmed that the retention of water diluent on the diethylamine stationary phase conducted to a compression of peaks eluting at the initial composition of 5 % MeOH (compounds N1-N3), as soon as 10 % water were added to the sample (Figure S1). The compression effect was related to the amount of water. Furthermore, when applying a gradient elution, this compression effect lasted as the amount of co-solvent increased. As shown in Figure 3, the extent of compression was directly linked to the retention of the analyte related to the water retention. The closer the analyte to the water molecules was located, the more compressed the peak was. This compression effect was also related to the amount of water that was injected alongside the low-retained analyte, down to a decrease in peak variance of 40 % when injecting in 90 % water.

On the other hand, the tag-along effect that broadened late-eluting peaks seemed to affect more significantly peaks with composition at elution over 30 % co-solvent, with variances reaching + 80 %, whereas for the test compound eluting at a composition of 18 % co-solvent, variance increased only by + 20 %.

The displacement/tag-along effect is generated by retained molecules that interfere with the retention of analytes. As MeOH was proved to be retained on the SFC column, the dilution of the analytes in a MeOH/water phase had a different impact as when ACN/water was the diluent. For low-retained compounds diluted in over 80 % MeOH, a strong solvent effect was present, due to the competition with the MeOH molecules from the diluent (Figure S2a, black marks) and the compression usually generated by water (Figure S2a, ACN/water diluents, white marks) was annihilated by using MeOH as

part of the diluent. The broadening observed for peaks eluting before water, when diluent was 10/90 MeOH/water, remains unexplained so far. Because MeOH exhibited a lower retention than water molecules, analyte peaks eluting after water underwent no competition with MeOH diluent molecules and hence their broadening was not significantly affected by the nature of the organic diluent (Figure S2b).

3.3 Analyte position relative to water peak

Since the water plug is beneficial for the analytes that migrate faster than water molecules, it may be tempting to modify the chromatographic conditions so that the retention of analytes becomes lower than that of the water. However, the retention of polar compounds in SFC usually follows the same trend and it was necessary to find a stationary phase for which the water retention trend (ln k vs. co-solvent) intersects with test compounds. Isocratic experiments were performed at co-solvent volumetric fractions ranging from 2 to 40 %, injecting test compounds N6-N9 surrounding the water peak to determine their retention on DEA, diol and HILIC stationary phases (Figure S3). Models expressed as volumetric fractions are easier to use but they are valid only for given operating conditions, here 40 °C, 140 bars at BPR. Any transfer of models would require translation of volumetric fraction into mass fraction of co-solvent [20]. The retention data were fitting a HILIC mixed-mode model for the three columns (Figure S3). The position of test compounds relative to water elution could be switched on HILIC column by changing the gradient slope. At a normalized gradient slope of 2 % (Figure 4a), N9 eluted after water (Ce 12.7 % MeOH vs. 9.3 % MeOH, respectively, calculated using first-order moment), whereas at 30 % normalized gradient slope (Figure 4b), N9 eluted before water (27.3 % and Ce 31.1 % MeOH, respectively). The faster elution resulted in an overall peak sharpening (Figure 4) as also observed in LC. For example, for N9 diluted in MtBE, the peak variance decreased from 600 µL² to 20 µL² for 2 % and 30% normalized gradient slopes, respectively. The effect of water in the diluent was hence normalized for each gradient slope to the variance when the diluent was MtBE. The change of position relative to the water peak confirmed the test compound was compressed if eluting before water molecules, as shown in Figure 4a and broadened if eluting after (figure 4b), with the extent of the phenomenon related to the water content in the diluent.

3.4 Influence of the co-solvent nature

Method development in SFC may require the use of different co-solvents to easily tune the separation. Methanol and acetonitrile are the most used co-solvents but mixture of both or ethanol are now gaining attention. As opposed to mobile phase components in LC, SFC co-solvent molecules are known to be prone to interaction with the stationary phase [21, 27]. The impact of the co-solvent nature was studied by comparing variances using MeOH, ACN, mixture of MeOH/ACN 50/50 and EtOH as co-solvent. Changing co-solvents did not affect the retention order of test compounds (Table S1) as polar molecules showed quite similar retention trends in SFC. Solutes injected in MtBE diluent were found to exhibit higher peak variance when the co-solvent was aprotic (ACN) than when it was protic (MeOH or EtOH) (Figure S4a). When the sample was diluted in a water-containing diluent, the trend in variance change was the same as described before, whatever the co-solvent. Solutes that

were less retained than water were subjected to two mechanisms: competition with the water diluent leading to peak compression, and competition with the co-solvent molecules to access the stationary phase. For a test compound eluting before water (white marks on Figure 5), it is clear that the competition from alcohol co-solvents (Figures 5a and 5b) was beneficial and the compression increased for peaks closer to water (here, N7). On the contrary, the injection of a water/ACN diluent in an aprotic co-solvent such as ACN (Figure 5c) led to broadening when the content of water was low, and to compression when the content of water in the diluent was elevated.

More surprisingly, the use of a mixture of ACN/MeOH 50:50 as a co-solvent, which is expected to generate less competition as less MeOH molecules compete with analytes, was highly compatible with the injection of water/ACN diluents, helping for the compression of early-eluting analyte peaks such as N1. This result has to be mitigated by the fact that MeOH/ACN co-solvent generated much larger peaks than any other co-solvent when injecting samples in apolar diluents (Figure S4a).

Finally, because the retention of the studied co-solvent was always lower than water molecules, analyzing compounds with large retention (grey marks in Figure 5) diluted in hydro-organic phases was not affected by the nature of the co-solvent used. Interestingly, Figure 5b suggests that injecting in a water-based diluent led to better results than injecting in MtBE when using an EtOH co-solvent.

3.5 Effect of additives in co-solvent

In recent years, water has been introduced as an additive in the organic co-solvent to enhance the mobile phase polarity range [28]. This addition of water, up to 7-8 % with methanol, exhibits a great opportunity for the separation of more polar compounds [29]. Another benefit of adding water to the co-solvent is its impact on peak shapes. Khvalbota et al. [30] reported an improvement in efficiency for neutral compounds isocratically separated on a Fructoshell-N stationary phase, as long as they were more retained than water. Moreover, efficiency was improved for neutral compounds on a silica column, whatever their relative positive towards water.

Adding 2 % water in MeOH co-solvent had very little yet positive effects on the gradient separation of neutral test compounds on the DEA stationary phase. Retention was slightly reduced (Table S1). When injection was performed using MtBE, the peak variance was improved for all compounds by 10 % - 20 % using water as an additive in co-solvent (Figure S4b). This behavior is similar to what was observed on the silica shell column by Khvaltova [30]. However, the beneficial effect of adding water to the co-solvent was reduced when the sample was also in an aqueous media. As can be seen in Figure 6, when comparing the absolute peak variance obtained when using no additive (Figure 6a) or when using water as an additive (Figure 6b), the trend was very similar. Here again, the compounds eluting before the water molecules were compressed, but the influence of the water content in the diluent acted to a lesser extent, the peak variance being already influenced by the water adsorbed on the stationary phase prior to injection. In addition, the tag-along phenomenon was also reduced, with the peak variance increasing by 40 % over the range of diluent composition, compared to 70% when using no additive. This may be due to the fact that the competition between analytes and water for the stationary phase lasted all along the gradient run and not just in the water injection plug. Hence the presence of water in the co-solvent tended to level the effects of water in the diluent, whether beneficial or detrimental.

In order to introduce an additive that would efficiently compete with the water molecules from the injection solvent, formic acid (FA) was investigated. Anionic additives are often used to improve peak shape when using stationary phases with a basic character such as the Torus DEA [31]. Formic acid can be used in SFC as an alternative to TFA as column regeneration is faster [32]. Formic acid was more retained than water on DEA stationary phase, with a Ce of 23.5 % MeOH. A concentration of 0.1 % FA in the methanol co-solvent did not affect the retention of neutral test compounds (Table S1). However, it broadened the peaks significantly for 7 out of 10 compounds using MtBE as diluent (Figure S4b). When comparing the absolute peak variances obtained when injecting in water-containing diluents, the addition of formic acid in the MeOH co-solvent improved the peak variances of retained compounds N8-N10 (Figure 6c), by a factor 30 % - 50 % compared to the use of pure MeOH as co-solvent, while it had a dramatic effect on less retained compounds N4-N7 with absolute peak variances increasing by a factor 3. Hence, formic acid as an additive to MeOH has to be avoided if MtBE is the sample diluent. Nonetheless it may be of interest for highly polar solutes diluted in ACN/water.

3.6 Ionizable solutes

The expansion of SFC applications towards more polar compounds such as metabolites, amino acids, nucleoside, peptides [2] pave the road towards the analysis of ionized molecules by LC x SFC. These highly polar analytes typically elute from LC in a great amount of water thus it seemed essential to check the behavior of ionized test compounds in this study.

West et al. stated using pH indicator that the apparent aqueous pH of carbon dioxide – methanol compositions should be around pH 5 with a decreased value when the proportion of methanol increased [33]. The selected acidic test compounds (pKa around 4.5) were supposed to be partially ionized. Interestingly, the peak variance of acidic compounds was not influenced by the content of water in the diluent (Figure 7).

With composition at elution over 20 % MeOH, the selected acidic test compounds A1-A3 were highly retained. However, no peak broadening was observed on these negative analytes, whatever the content of water in the diluent while neutral compounds eluting at the same composition (N4 and N8) suffered an increase in peak width which extent was linked to the water content in the diluent, as previously discussed.

On the other hand, two basic test compounds B1 and B2 were analyzed, B1 with a lower retention than water, and B2 with a larger retention than water. Ammonium hydroxide was added to the co-solvent to improve peak symmetry [14]. Selected test compounds had both a pKa at 9.4 and so the assumption can be made that they were fully protonated all along the gradient run. Unlike for other test compounds, MtBE as an injection solvent was found to provide symmetrical yet very wide peaks. Hence a mixture of ACN/water 90:10 was used as the injection solvent of reference for the calculation of variance change. The general trend for basic solutes followed what was observed for neutral solutes, with a peak compression for compounds eluting before the water peak and an increase in peak width for compounds more retained than water (Figure 7b). The compression on low-retained B1 peak ranged between -5 % and – 20 % depending on the water content, which was very close to the value observed for the neutral test compound N4 eluting at the same composition. On the other hand, the selected retained basic compound B2 eluted at Ce 15 % MeOH which was very close to the water

peak. The increase in peak width that B2 underwent was of a much larger extent than the one observed for the neutral test compound N8 eluting at its vicinity.

While these results need to be confirmed with a much larger pool of ionizable compounds, it seems that in the case of our selected test compounds, negatively charged solutes and positively charged solutes exhibiting a lower retention than water molecules were not influenced by the water content in the injection solvent. On the contrary, the presence of water-rich solvent was detrimental for the positively-charged compounds with a retention close to water.

Adding 2 % water to the co-solvent was positive for the peak shapes of basic compounds when the injection solvent was of low polarity such as MtBE, as previously stated by West and Lemasson [34], but it was found highly detrimental when injecting in a water-based phase (Figure S5a). Injecting water in a water-containing co-solvent led to the formation of a perturbation peak at 2.5 min and a tracer peak at 3.4 min, visible at 200 nm (Figure S5b). With water content as low as 10 % in the diluent, the peaks of basic compounds eluting before the perturbation peak were splitting towards higher retention. The peaks with higher retention kept symmetrical shape up to 30 % water in the diluent, then when increasing the amount of water in the diluent, the peaks also splitted towards higher retention. In comparison, acidic or neutral compounds never underwent any splitting in the same conditions.

3.7 Increasing the injection volume

Desfontaines et al. [1] stated that injection of samples with high content of water was possible for low injection volume (< 2 µL) while the injection of 10 µL (2 % column volume) conducted to a demixing when using an initial mobile phase containing only 2 % co-solvent (MeOH/water 98:2 + 0.1 % ammonium hydroxide). The solubility of water is indeed very low in supercritical CO_2 (~0.1% w/w). Increasing the initial mobile phase composition to 5 % MeOH increased the solubility of water and during the course of our experiments, a volume of 10 µL of a sample diluted in pure water could be injected on a column of similar geometry (100 x 3.0 mm, 1.7 µm) without demixing issue. Increasing the injection volume from 5 µL to 10 µL led to an expected peak broadening but the peaks remained symmetrical, and retention times and system pressure kept unchanged. The peak capacity when injecting in water-containing diluents remained above 75 % of the peak capacity observed when injecting 10 µL sample in MtBE. Increasing the injection volume to 20 µL (4 % column volume) led to two increases in the profile of the system pressure (Figure S6a). The first one occurred during the elution of MeOH molecules while the second one occurred during the elution of water molecules. Since both increases were proportional to the amount of water injected in the column, we suggest they were due to the local increase of viscosity generated by 1) the displaced MeOH molecules and 2) the water plug. Pressure values were back to normal as soon as water eluted out of the column. While peaks were broadening and splitting with an increase content of water in the diluent, injecting 90% water diluent led to a massively distorted broad peak for compounds eluting at the vicinity of water molecules (Figure 8). However, the system peak due to water was visible at the same time at it was when injecting 5 µL (Figure S6b) and the UV signal was not disrupted, suggesting that water plug still eluted at the same velocity and demixing was not happening. A better understanding of water

interaction with stationary phase and solutes in SFC would be required before injecting such amount of water diluent.

On the other hand, neutral test compounds eluting at the end of the gradient, such as N10, exhibited fronting peaks while peaks from acidic test compounds with the same retention time remained symmetrical with no retention shift (Figure 8). Supposedly, ionic interactions with the stationary phase in SFC played a role in maintaining the peak symmetry for acidic test compounds in this study, but this point needs a deeper investigation with a larger set of ionizable compounds.

4. Conclusion

The composition of the sample diluent has a major role in the peak broadening in SFC. While previous papers highlighted the strong solvent effect due to polar solvents such as alcohols, this work focuses on the injection of water-based diluents. Peak broadening of 10 test compounds were compared when injected either in MtBE or in diluents containing up to 90 % water. Statements that water has a negative effect on peak shape when it is part of the diluent is only partly true. When injected simultaneously with neutral analytes, water plays a competing role for the SFC stationary phase, leading to a compression of peaks that elute before water, and a broadening of peaks that elute after water. The phenomenon is related to 1) the retention of analytes in regards to the retention of water and 2) the amount of water in the diluent. It has been confirmed on various stationary phases. Adding water or acidic additive to the co-solvent did not affect this process for neutral analytes. The presence of water along acidic analytes in the sample had no effect on their peak shapes, while for basic compounds, it led to dramatic peak deformation. Moreover it was possible to inject 10 µL of samples in water-containing diluents (2 % column volume) without major peak capacity loss, while increasing the injection volume up to 20 µL had negative consequences on peak shapes of neutral molecules. In the light of these results, the analysis of polar compounds diluted in hydro-organic mixtures, or the use of SFC as second dimension in a two-dimensional separation of neutral compounds could be considered without requiring sample water removal, providing that injection volume remained below 2 % of the column volume. Interestingly, acidic compounds can hold very large injection volumes with water-rich diluents, while the analysis of basic compounds seems problematic even at very small injection volume. While this study highlighted clear trends for neutral compounds, a deeper investigation will be required for ionizable compounds.

5. Acknowledgments

The authors are deeply grateful to Agilent Technologies for the loan of the Infinity I fixed-loop injection module and especially Audrey Menet for her technical advices. Marion Burlet-Parendel is acknowledged for preliminary results.

6. References

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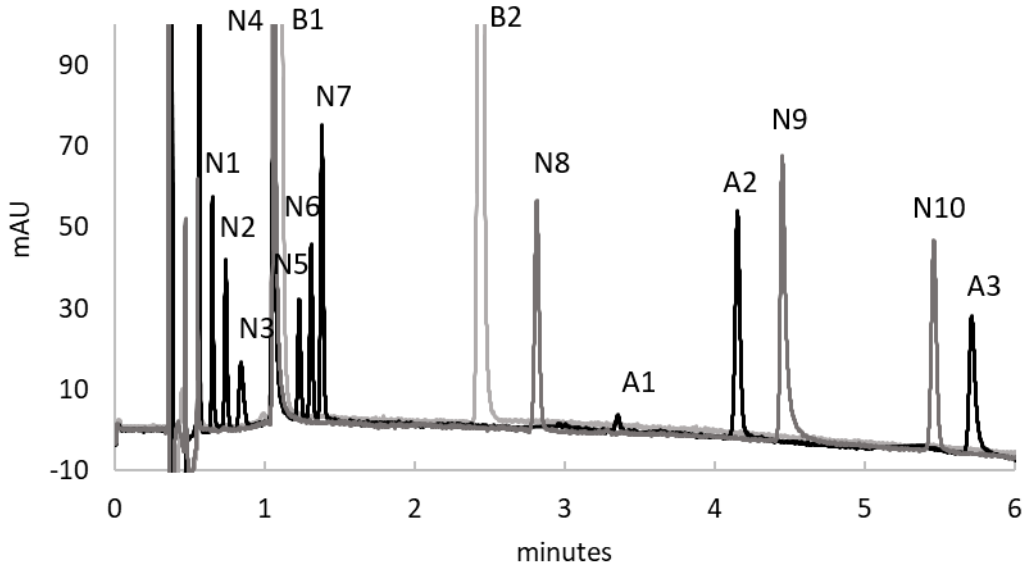
Table 1: Selected test compounds and associated retention time and composition at elution using DEA column. MeOH as co-solvent for neutral and acidic compounds, MeOH + 2 % water + 20 mM ammonium hydroxide for basic compounds.

		Probe number	log P	tr (min)	Ce (%)
Neutral	eugenol	N1	2.223	0.65	5.0
	2.4.6 trimethylphenol	N2	1.7	0.74	5.0
	1-indanol	N3	1.518	0.84	5.0
	apocynin	N4	0.77	1.05	6.3
	o-cresol	N5	1.95	1.23	7.5
	m-cresol	N6	1.96	1.31	8.0
	phenol	N7	1.46	1.38	8.5
	4-hydroxybenzyl alcohol	N8	-0.121	2.81	17.9
	naringenin	N9	0.79	4.45	28.7
	arbutin	N10	-0.652	5.45	35.3
Acidic	acrylic acid	A1	0.35	3.35	21.4
	trans cinnamic acid	A2	1.887	4.15	26.7
	ferulic acid	A3	0.78	5.71	37.0
Basic	imipramine	B1	3.112	1.08	6.4
	propranolol	B2	2.535	2.44	15.4



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565

566 Figure 1: Separation of the 15 test compounds. Column DEA. Co-solvent MeOH for neutral and acidic
567 compounds (N1-N10; A1-A3) and co-solvent MeOH + 2 % water + 20 mM ammonium hydroxide for
568 basic compounds (B1-B2). Injection 5 µL in MtBE diluent. Detection 220 nm. Test compounds
569 description: refer to table 1.

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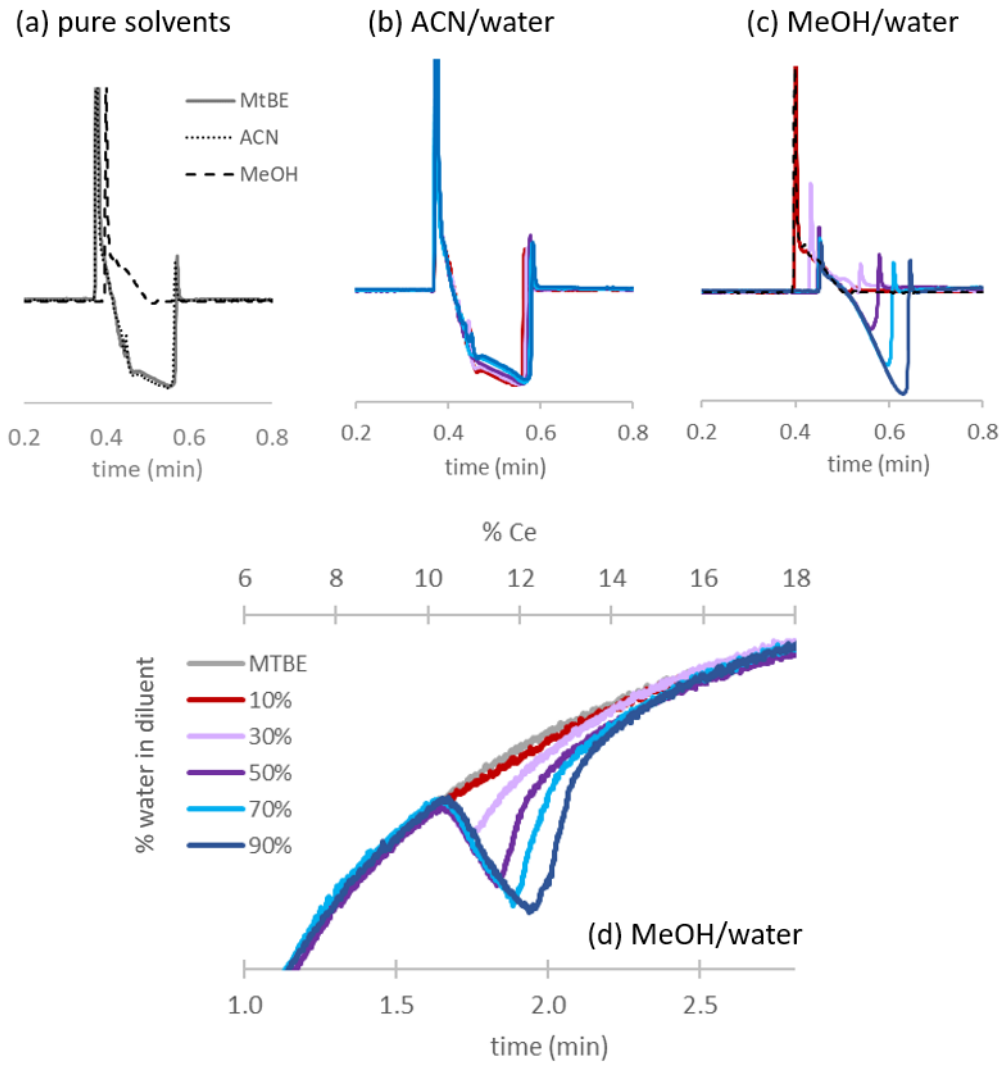

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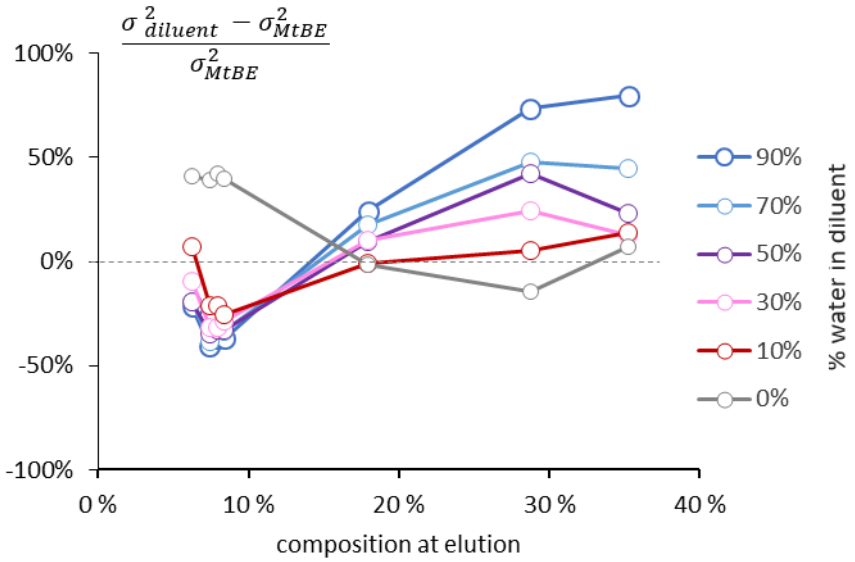
573 Figure 2: Chromatograms corresponding to the injection of (a) pure diluents: MtBE (grey line), ACN
574 (dotted line) and MeOH (dashed line), (b) ACN/water mixtures and (c, d) MeOH/water mixtures. The
575 colored lines correspond to increasing amounts of water in diluent, from 10 % (red line) to 90 % (blue
576 line). Column DEA. Co-solvent MeOH. Injection volume 5 µL. Detection at 200 nm.
577



578

579 Figure 3: Diluent effect reported as change in peak variance vs. the composition at elution of neutral
580 test compounds, for various percentages of water in the ACN: water diluent. Reference variance from
581 the injection of test compounds using MtBE as diluent, with conditions as in Figure 1.

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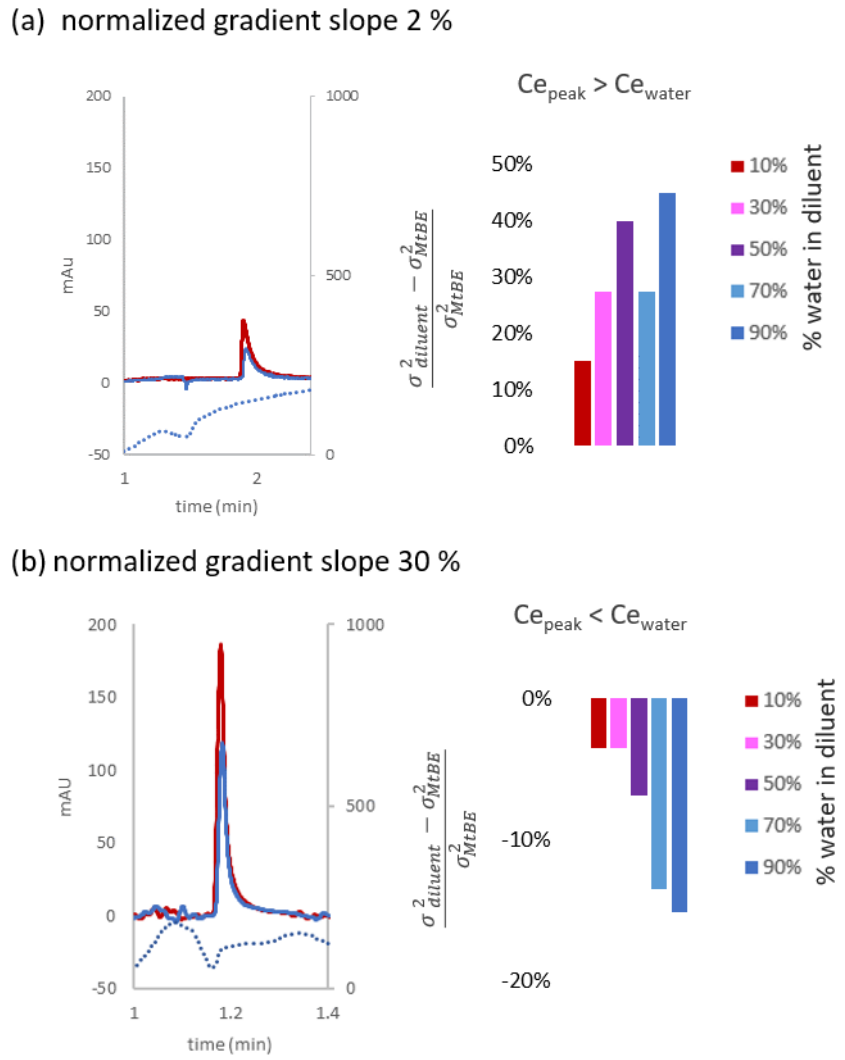

584

585 Figure 4: Chromatograms of N9 (plain line, 220 nm) and water (dashed line, 200 nm) and associated
586 injection solvent effects, for a) a normalized gradient slope of 2 % and b) a normalized gradient slope
587 of 30 %. HILIC column, gradient 5 % to 40 % MeOH.

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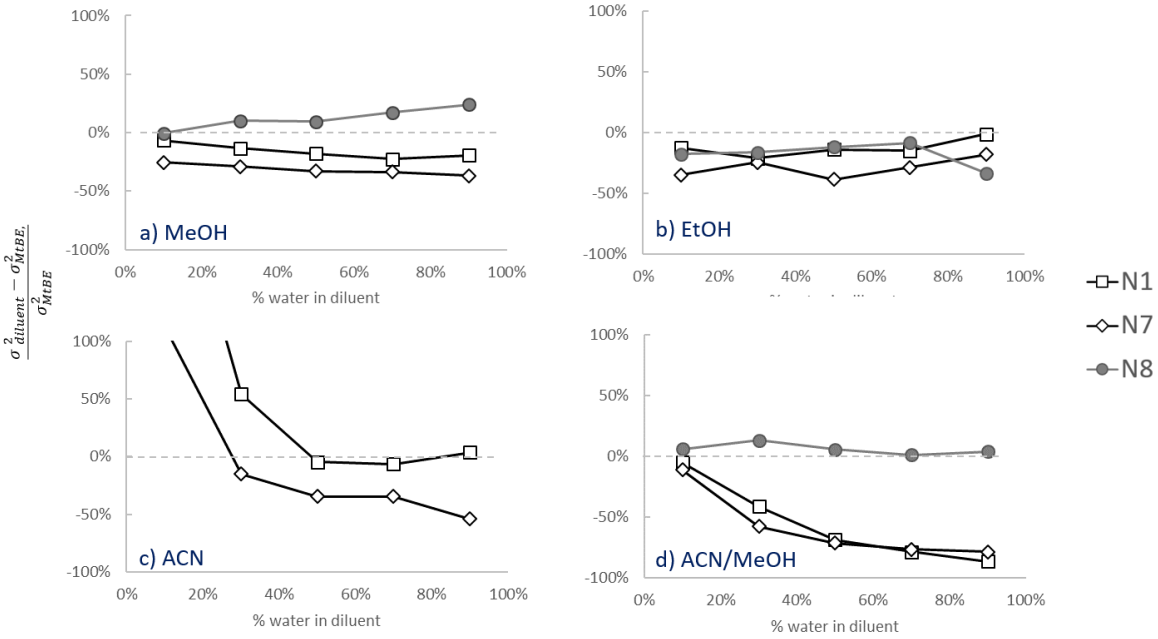

Figure 5: Influence of the co-solvent on the change in peak variance vs. the content of water in the ACN/H_2O diluent, for co-solvent (a) MeOH, (b) ACN, (c) EtOH and (d) MeOH/ACN 50/50. Compounds N1-N7 (black marks) elute before water, N8 (grey marks) after water. Please refer to Table S1 for the values of composition at elution in the different co-solvents.

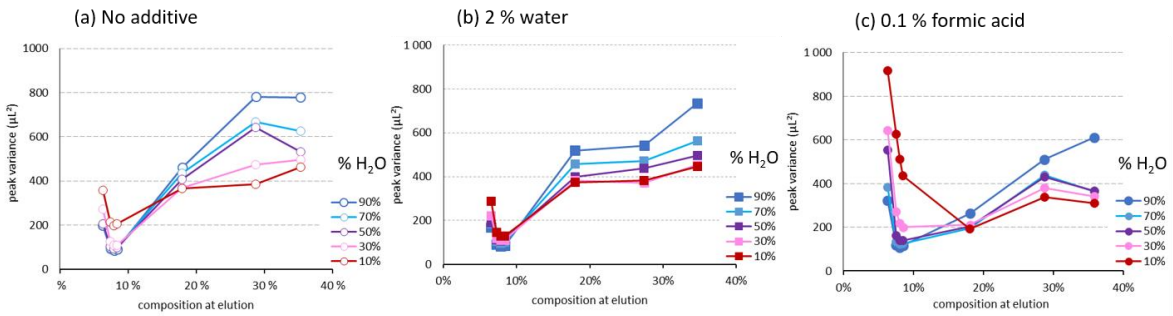

Figure 6: Effect of additives in modifier on the peak variance for various water content in the ACN/water diluent. Mobile phase CO_2 with the co-solvent (a) MeOH, (b) MeOH +2 % v/v water and (c) MeOH + 0.1 % w/w formic acid

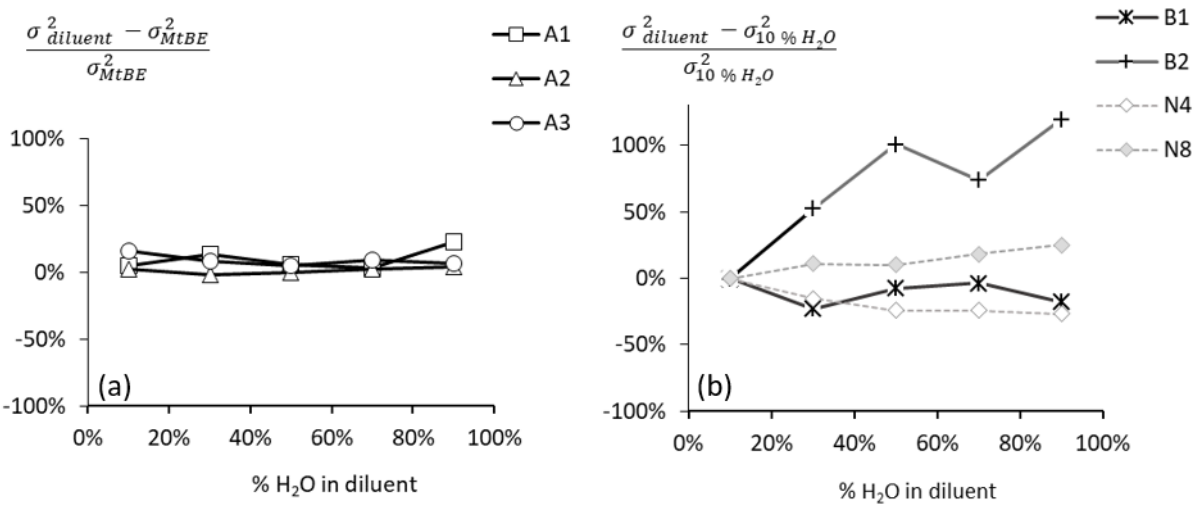

Figure 7: Diluent effect reported as change in peak variance vs. the content of water in the diluent. (a) acidic test compounds A1-A3, with MtBE diluent as reference and (b) basic test compounds B1-B2 with ACN/water 90:10 diluent as reference. The dashed lines represent test compounds N4 (white diamond) and N8 (grey diamond).

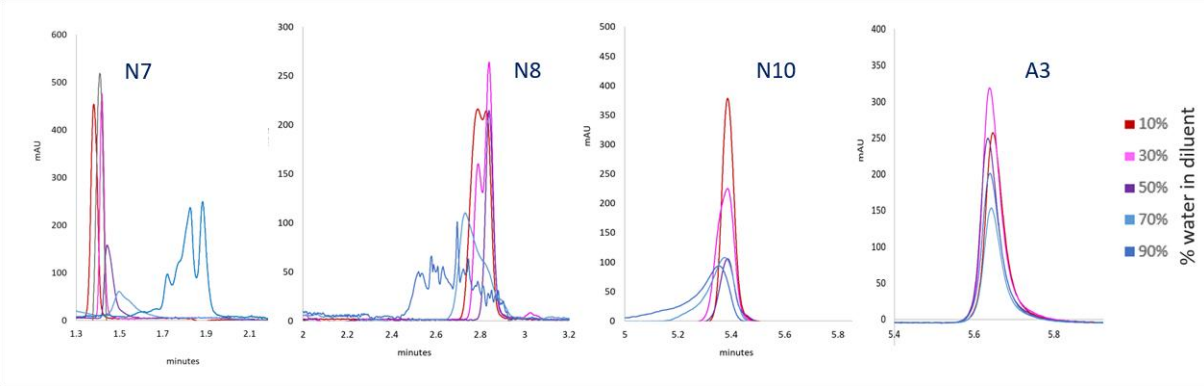

Figure 8. Chromatograms of three neutral probes and one acidic probe. Injection 20 µL. Column DEA. Co-solvent MeOH. UV detection 220 nm. Other conditions as stated in the text.

Supplementary material

Effect of the injection of water-containing diluents on band broadening in analytical supercritical fluid chromatography

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List of supplementary material

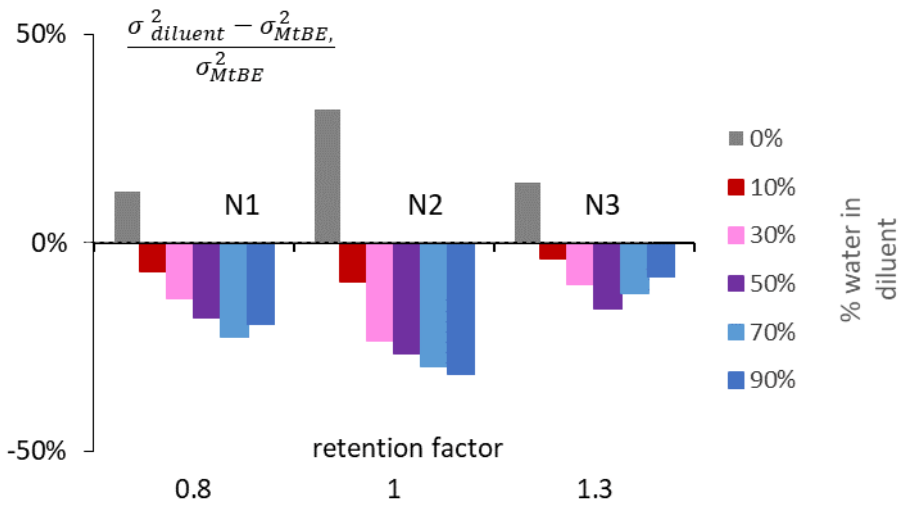

Figure S1: Change in peak variance for peaks N1-N3 eluting at initial mobile phase composition, for various percentages of water in the ACN/ water diluent. Column DEA, co-solvent MeOH.

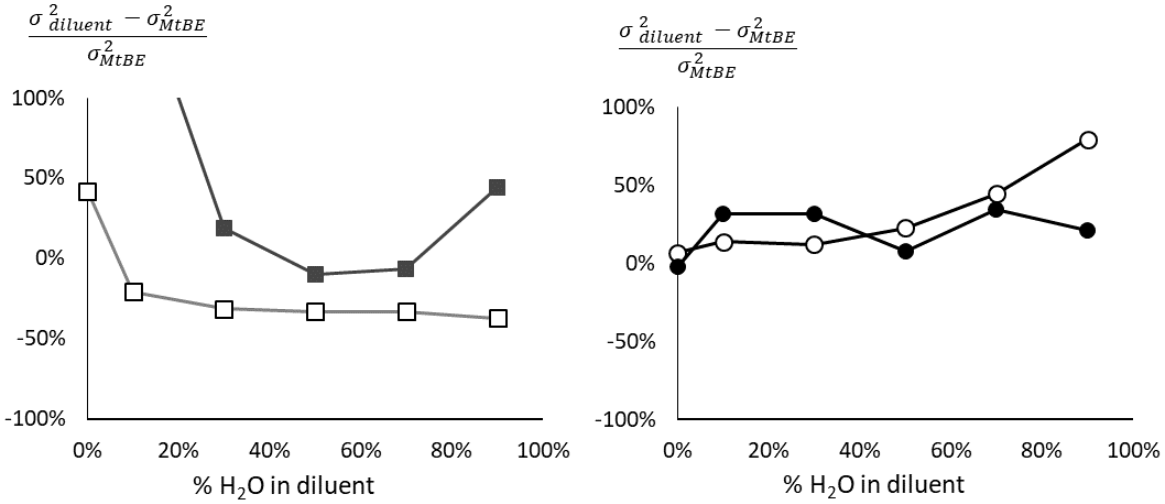

Figure S2: Injection solvent effect reported as change in peak variance for (a) N6 and (b) N10 vs. the content of water in the diluent, the remaining solvent being acetonitrile (white marks) or methanol (black marks).

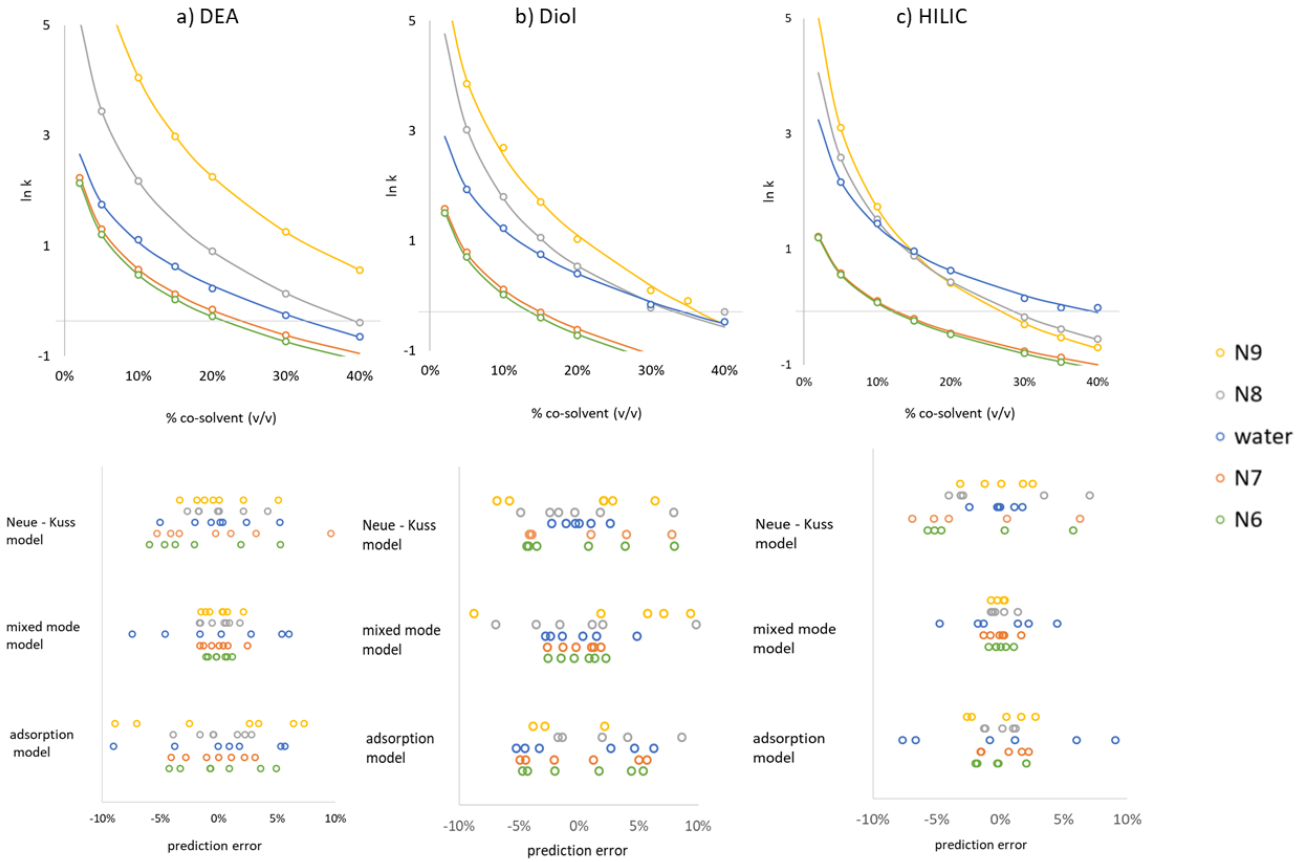

Figure S3: Retention relationships of water and four surrounding probes N6-N9. Ln k as a function of the MeOH content in mobile phase. Circles are experimental measurements based on isocratic runs. Plain lines are the associated prediction using mixed-mode model. Below are the associated prediction errors of three models. Percentage errors defined as $(k_{predicted} - k_{exp})/k_{exp}$. Columns (a) Torus DEA, (b) Torus diol and (c) BEH HILIC.

Equations for

Neue-Kuss model $\ln k = \ln k_0 + 2\ln(1 + S_2\,\varphi) - \frac{S_1\varphi}{1+S_2\varphi}$

Mixed-mode model $\ln k = \ln k_0 + S_1\varphi + S_2 \ln \varphi$

Adsorption model $\ln k = \ln k_0 - S_1 \ln \varphi$

where ϕ is the volumetric fraction of co-solvent, k_0 the extrapolated value of k for ϕ = 0 (i.e., pure CO_2), S1 the slope and S2 the curvature coefficient.

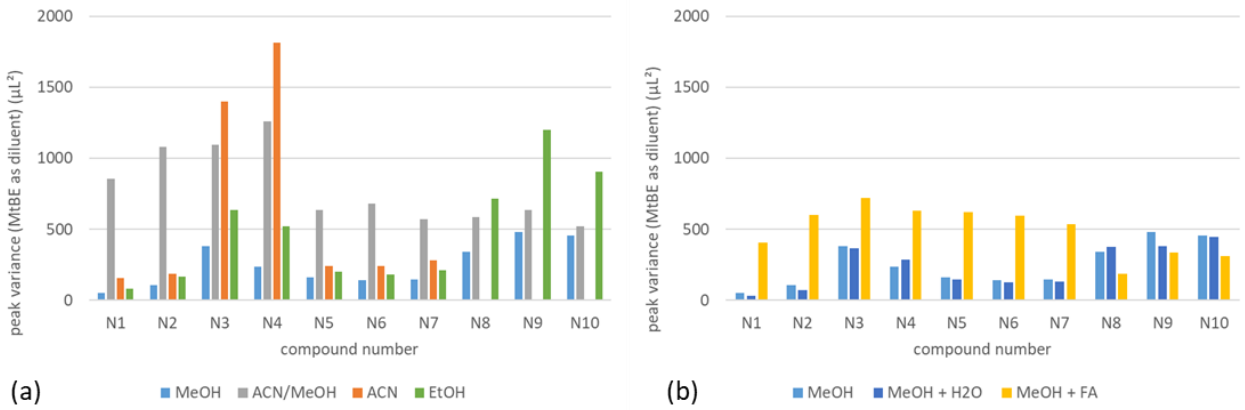

Figure S4: Peak variance of the neutral test compounds, using MtBE as diluent, (a) for various organic co-solvents and (b) using MeOH with additives, i.e. 2 % water or 0.1 % formic acid additives as co-solvent. Compounds N8-N10 were not eluting during the gradient when using ACN as co-solvent.

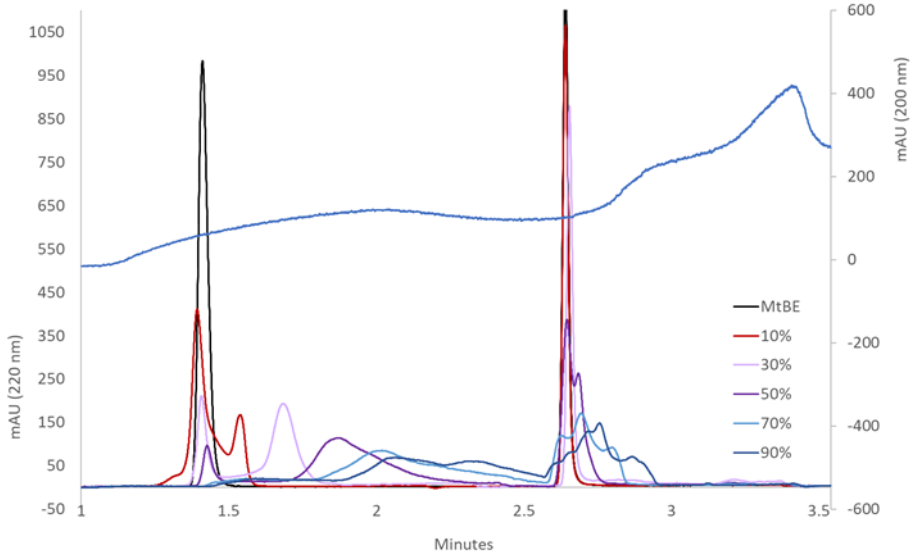

Figure S5: (a) Chromatograms of two basic probes injected in ACN/water diluent with increasing content of water. UV detection 220 nm. (b) UV trace of pure water. UV detection 200 nm. Column DEA. Co-solvent MeOH + 2 % water + 20 mM ammonium hydroxide.

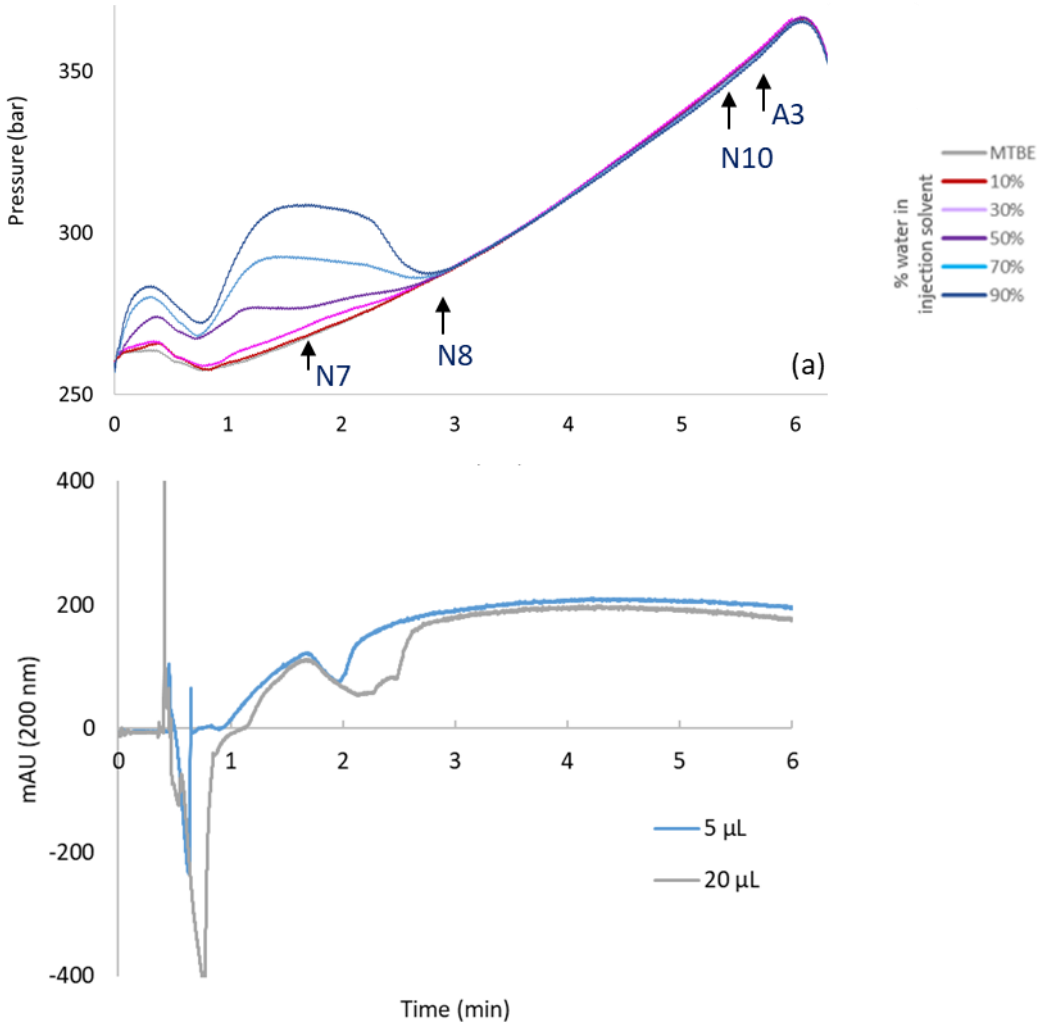

Figure S6: a) SFC inlet pressure when injecting 20 µL ACN/water with increasing content of water and b) System peaks recorded at 200 nm, when injecting 5 µL and 20 µL pure water. Column DEA. Co-solvent MeOH. Other conditions stated in text.

Table S1: Selected test compounds and associated composition at elution for different co-solvents. Column DEA, gradient 5 % to 50 % co-solvent, normalized gradient slope 2 %.

	Compound		Composition at elution (% co-solvent) MeOH	ACN/MeOH 50/50	ACN	EtOH	MeOH + 2 % H_2O	MeOH + 0.1 % FA
Neutral	eugenol	N1	5.0	5.0	5.0	5.0	5.0	5.0
	2.4.6 trimethylphenol	N2	5.0	5.0	6	5.0	5.0	5.0
	1-indanol	N3	5.0	5.7	7	5.6	5.0	5.0
	apocynin	N4	6.3	7.1	11	7.4	6.5	6.3
	o-cresol	N5	7.5	8.7	14	8.8	7.3	7.5
	m-cresol	N6	8.0	9.5	16	9.4	7.8	8.1
	phenol	N7	8.5	9.9	17	9.8	8.4	8.5
	4-hydroxybenzyl alcohol	N8	17.9	36.6	21.2	20.5	18.0	18.1
	naringenin	N9	28.7	Over	31.8	32.0	27.4	28.7
	arbutin	N10	35.3	Over	42.2	38.9	34.7	35.9
Acidic	acrylic acid	A1	21.4	29.7	Over	31.3	20.8	17.4
	trans cinnamic acid	A2	26.7	34.8	Over	37.6	25.2	19.1
	ferulic acid	A3	37.0	43.6	Over	Over	35.8	29.6
Basic	imipramine	B1	6.4	N.D.	N.D.	N.D.	N.D.	N.D.
	propranolol	B2	15.4	N.D.	N.D.	N.D.	N.D.	N.D.
	Water		13.7	13.5	N.D.*	15.6	13.9	12.3

N.D.: not determined

Over: retention over the gradient range (> 50 % co-solvent)

*The water peak could not be detected at 200 nm due to the lack of UV absorbance of ACN co-solvent.