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Review

Packed column supercritical fluid chromatography of hydrophilic analytes via water-rich modifiers

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ABSTRACT

The use of additives to dramatically extend the range of solute polarity amenable to CO_2 based supercritical fluid chromatography (pcSFC) was predicted over 20 years ago. At that time additives were predicted to have multiple functions such as enhancement of mobile phase solvating power, ion suppression, and ion pairing. The adsorption of mobile phase components on the stationary phase causing a modification of its surface was predicted, but the implications for separations were not defined. Reports published in the late 1980s showed that while water could not function as a primary modifier due to it poor solubility in carbon dioxide, its use as an additive was more promising. The past decade has seen very little published work concerning water and pcSFC. Now reports are beginning to appear that demonstrate enhanced selectivity with water, and application of the technology to polypeptide salts, drug molecules, and nucleobases. This review attempts to bridge the past with the present. As evidenced by the studies described in this review, water may offer much potential as an additive in that it could (a) enhance the solvating power of the mobile phase, (b) introduce HILIC-like analyte partitioning, (c) simplify preparative purifications, and (d) offer a more mass spectrometrically compatible interface.

Contents

1. Introduction

There are relatively few references in the literature concerning packed column supercritical fluid chromatography (pcSFC) that deal with the incorporation of water into a carbon dioxide-based (CO_2) mobile phase. Addition of water to the mobile phase might improve peak shapes and/or extend the polarity range of pcSFC to more polar analytes. Furthermore, water does not have some of the objectionable characteristics of organic solvent modifiers such as UV absorbance. While an aqueous CO_2-based fluid remains compatible with the universal flame ionization detector, the degree of improvement and the range of additional solutes that has been helped in the past have been marginal. Work during the past 5–6 years with water and pcSFC offers interesting possibilities for the

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future. Clearly a systematic, detailed evaluation would do much to clarify the role water plays in pcSFC. Hopefully, this non-exhaustive review can aid in this scientific endeavor which should readily reach into the pharmaceutical and food areas.

2. pcSFC defined

Packed column SFC (pcSFC) in the 21st century is probably better described as "carbon dioxide-based HPLC". It has become more widely accepted and increasingly popular because of its ability to solve problems that deal with highly polar analytes, and to the new user it "feels and looks" like HPLC. In addition, several practical characteristics of carbon dioxide (CO_2) cause pcSFC to be faster, more scalable, and greener. CO_2 is sold as a liquid in equilibrium with a gas in high pressure gas cylinders. Near room temperature, the pressure in the tank is approximately 60 bar. A pump is required to boost the pressure as high as 400–600 bar. Virtually all pcSFC is performed using CO_2-based fluids due mostly to the lack of better, more polar alternatives. Most pcSFC is performed at modest temperatures near the critical temperature of the fluid. The operator cannot tell when the definition, or the name, of the fluid changes from subcritical to supercritical. Under most practical conditions, the technique called pcSFC is actually an odd form of high performance liquid chromatography (HPLC) with a highly compressible solvent. "Carbon dioxide-based HPLC" adequately describes the technology and is, in fact, a name that has previously been suggested for pcSFC although the term never has "caught on" [1]. pcSFC is actually a misnomer since the characteristics of the chromatography that are exploited (high diffusivity and low viscosity) are present irrespective of whether the fluid is supercritical or just a subcritical liquid.

pcSFC is a normal phase technique without the problems usually associated with normal phase HPLC. Practical problems associated with the latter include (a) slow equilibration times, (b) difficulty in running gradients, (c) traces of water in the solvents, and (d) flammable waste. In normal phase HPLC, the least polar solutes tend to emerge first which is roughly opposite to reversed phase HPLC. Normal phase chromatography which traditionally used mostly hexane with a slightly more polar modifier and a polar stationary phase such as bare silica is seldom practiced nowadays. CO_2 is no more polar than hexane. Polar solutes are soluble only at the ppb and ppm levels [2,3]. Its solvent strength can be adjusted by mixing small volumes of polar organic solvents with the main fluid. Modern pcSFC almost invariably requires modified mobile phases to cover a wide range of solute polarity. When a modifier is added, pressure becomes a secondary control variable [4]. There is seldom any compelling reason to raise the temperature much above ambient. As in HPLC, the primary mode for screening a wide range of solute polarity involves composition gradient programming.

3. Role of modifier and additive

Methanol is probably the most polar, common modifier completely miscible with CO_2 over a wide range of temperatures and pressures. In pcSFC screening methods, one can program from very low modifier concentrations (i.e. 2–5%) to very high modifier concentrations (i.e. 50–60%). Upon increasing the modifier concentration, the critical temperature and critical pressure of the mixture are likely to increase. If the operating temperature is below the critical temperature of the new mixture, the definition of the fluid changes to a liquid. However, in almost all practical circumstances, it is irrelevant whether these fluids are defined as subcritical or supercritical. No significant changes in either physical or chemical mobile phase characteristics occur when the definition of the fluid changes [5]. In addition to the mobile phase and solute, modifier also interacts with the stationary phase [6,7]. The proportion of adsorbed modifier onto the stationary phase depends on the mobile phase composition. In practically all situations the amount of adsorbed modifier was greater than the modifier percentage in the bulk mobile phase. Working with methanol/CO_2 (2/98), the percentage of methanol in the stationary phase was equal to 40% [8]. Lesellier states that this stationary phase polarity modification acts on (a) compound retention, (b) the apparent magnitude of the void volume, and (c) it complicates chromatographic modeling.

A major advance in pcSFC occurred with the introduction of a third more polar component (i.e. additive) into the mobile phase such as trifluoroacetic acid and isopropyl amine [9]. As suppression of ionization was believed to be the role of the additive, organic acids were used to elute acidic analytes and organic bases were used to elute basic analytes. More recently salts such as ammonium acetate have proven to be excellent additives for elution of ionic analytes thereby revealing an ion pairing strategy for pcSFC [10,11]. Without the use of additives, the most polar members of some families either tailed badly or failed to elute. With inclusion of additives into the mobile phase virtually all members of the families eluted with similar peak shape. Additives are now known to provide the key to the separation of more polar solutes via pcSFC.

Today, additional roles have been suggested and demonstrated for additives such as (a) coverage of active sites, (b) change polarity of the stationary phase, and (c) change polarity of the mobile phase. Efficiency is always nearly improved by the presence of additives under supercritical conditions as opposed to subcritical conditions [12]. Surface molar excesses of additives in addition to modifier were thought to play a large part in the resulting character of the pcSFC system. Revelations such as these have prompted new thinking concerning additive strategy for pcSFC of hydrophilic compounds [13]. In this regard, neutral water as either CO_2 modifier or additive presents both interesting potential and problems. Water has very low solubility (~0.1%, w/w) in supercritical CO_2 [14], but water along with CO_2 are miscible with methanol. Water is more polar and possesses twice the hydrogen-bonding capacity of methanol. Water in contact with CO_2 becomes acidic due to the formation and dissociation of carbonic acid [15]. The pH of water has been found to vary from 2.80 to 2.95 when measured under pressures of 70–200 atm and temperatures of 25–75 °C [16].

4. Water as modifier and additive – early studies

The employment of water with CO_2 for pcSFC has not been widely studied, but it can hardly be considered to be novel. Saturation of supercritical CO_2 with water was reported to improve significantly the resolution of long-chain free fatty acids with flame ionization detection approximately 25 years ago [17]. Early reports of equipment for saturating CO_2 have described the use of a silica gel pre-column inserted between the pump and the sample injection port. The silica gel was saturated with about 40% (w/w) water at room temperature. As CO_2 passed through the pre-column at 25 °C, water was desorbed thereby saturating the CO_2 an estimated 0.25 mol.% water at 1800–5500 psi pressure. The saturation was later performed above the boiling point of water to increase the amount of water that could be loaded homogeneously into CO_2 to 2.5–3.0% [18]. A number of applications of this dated technology have been reported. Engelhardt et al. showed that the addition of water significantly improved the peak shape of weak acids and bases on silica-based columns [19]. France et al. used an octyl column with water saturated CO_2 for the analysis of fatty acids along with di- and tri-glycerides [20]. More recently, vitamins were well separated with water-modified CO_2 [21,22]. A serious disadvantage with these systems was the amount of water dissolved in the

mobile phase varied as mobile phase passed through the saturator column, and it was difficult to control.

The use of water as an additive as opposed to a modifier wherein the water concentration can be higher and more accurately controlled has been more effective. Small concentrations (i.e. 10^{-4} M) of highly polar additives, however, cannot be added directly to nonpolar supercritical fluids. Instead they are added to a modifier of intermediate polarity such as methanol. In this regard, the chromatographic properties of ten mobile phase additives including water have been evaluated with respect to retention, selectivity, and efficiency [23]. Ten volume percent methanol was used both as a modifier and as a carrier for the additive under subcritical and supercritical conditions. Neutral additives such as water proved to be of little interest in the study as they were reported to not improve chromatographic efficiency in contrast to Bronsted acidic and basic additives. These results, however, are inconsistent with earlier reports which have demonstrated that methanol/water/CO_2 facilitate hydrophilic solute separations in pcSFC [24]. This report was also the first demonstration of the separation of enantiomers by SFC.

4.1. Underivatized amino acids

Two early papers in 1992 and 1997 stand out in this respect. Subcritical fluid chromatography coupled with evaporative light scattering detection was reported to allow the separation of underivatized amino acids on diol-bonded silica [25]. A mixture of methanol/water/triethylamine/pyridine (87.95/7.0/0.05/5.0, v/v) was chosen as the modifier. Pyridine could be substituted with ethylene glycol. Both were shown to impregnate the stationary phase and to improve efficiency. Samples containing 5–6 amino acids were partially separated.

4.2. Polysaccharides and polyols

The later more detailed report concerned the separation of monosaccharides and polyol using bare silica and trimethylsilyl (TMS) columns [26]. By adjusting column temperature to 60 °C and the flow rate to 5 mL/min, a complete separation of eight monosaccharides and polyols were obtained in less than 10 min. Mobile phase eluents of CO_2 at 200 bar with either 20% methanol, methanol/water, or methanol/water/triethylamine were studied. The content of water, presence of basic additive, and temperature had a major effect on capacity, selectivity, and efficiency. The maximum water content in the modifier was 9% without phase separation of the mobile phase. Efficiency on the TMS column was higher than on the bare silica column. The capacity factors of carbohydrates and polyols increased as the water content increased. Retention was suggested to be via a partition mechanism wherein the stationary phase was solvated by the hydro-organic modifier. An adsorption mechanism was not considered because the amount of polar solvent was too high.

4.3. Enhanced fluidity

Another more extensive series of papers which were initiated during the early 1990s should be mentioned here if only because the mobile phase contained methanol, water, and CO_2 albeit in much different proportions than previously discussed [27]. Such mixtures were termed enhanced fluidity liquid mixtures as they provide the advantages of high solvent strength and fast mass transfer. The liquid mixtures maintain the approximate strength of the associated liquid even when the mixture contains 50% CO_2. Although the maximum proportion of CO_2 here never exceeded 20 mol.%, the advantages of improved separation efficiency, increased optimum linear velocity, lower pressure drop, and decreased analysis time were observed [28]. The pH variation of enhanced fluidity liquid mixtures in which both H_2O and CO_2 are present has received limited study [29]. Clearly, the relationship between methanol/water modified-CO_2 where CO_2 is in excess and CO_2-modified methanol/water where the binary components are in excess should be further addressed.

5. Water as additive – today

5.1. Nucleobases

Serious consideration of water as a neutral additive for achiral pcSFC is again receiving much attention after more than a decade. The most useful and direct means of accomplishing this task is blending water as a tertiary component into an alcohol modified supercritical CO_2 mobile phase. The high polarity of water and its ability to function both as a hydrogen bond acceptor and a hydrogen bond donor is now recognized to enhance its role as an additive in packed column SFC. As a more recent example, the chromatographic effect of water as an additive with various alcohol-modified CO_2 mobile phases for the separation of several nucleobases on three widely utilized polar stationary phases has been reported [30]. The alcohols were methanol, ethanol, 1-propanol, and 1-butanol. The representative highly water soluble purine/pyrimidine analytes were thymine (4 g/L at 25 °C), uracil (0.4 g/100 g at 25 °C), adenine (0.5 g/L at 25 °C), and cytosine (1 g/130 g at 25 °C). Incorporation of a fixed amount of water additive into the alcohol modifier yielded markedly improved chromatographic traces for separation of nucleobases. With cyanopropyl, an increase in water percentage was accompanied by a slight decrease in retention time and a striking improvement in peak shape and intensity for adenine and cytosine. Thymine and uracil were found to yield relatively sharp peaks with or without water additive. These trends were also observed with the diol and pyridine phases in conjunction with both methanol and ethanol as modifier. While it was obvious that water as an additive was an asset for the separation of these four highly water soluble analytes, it was unclear how effective water would be in comparison with other commonly used additives.

Next 5% water was compared with 5 mM ammonium acetate (AA) as an additive wherein methanol or ethanol was the modifier. The employment of 25 mM AA had to be aborted since the addition of 5% water to the alcohol-AA modifier mixture caused the column to repeatedly plug. Fig. 1 captures the results of this study with ethanol modifier and the 2-ethyl pyridine phase. Reading from the bottom of the page with the understanding that the column was equilibrated with pure methanol for 25 min between runs, introduction of either 5 mM AA or 5% water or 5 mM AA + 5% water to the methanol modifier each yielded sharp, Gaussian peaks for each of the four components. The highly stable-UV detector response suggested the absence of a two-phase system resulting either from incompatibility of the polar and nonpolar components in the mobile phase or a transition to sub-critical conditions during gradient delivery of the modifier. It is important to note that the back pressure regulator temperature was not increased to prevent ice formation at the outlet in this study. The superior chromatography exhibited with water as the additive and the equivalency of water and ammonium acetate indicate that water as a neutral additive may have unique advantages for detectors where mobile phase elimination is desirable such as flame-based detectors.

5.2. MeOH vs. IPA as modifier

Thurbide et al. have noted that the maximum methanol/water ratio that can be tolerated in supercritical CO_2 is ~9/1 [31].

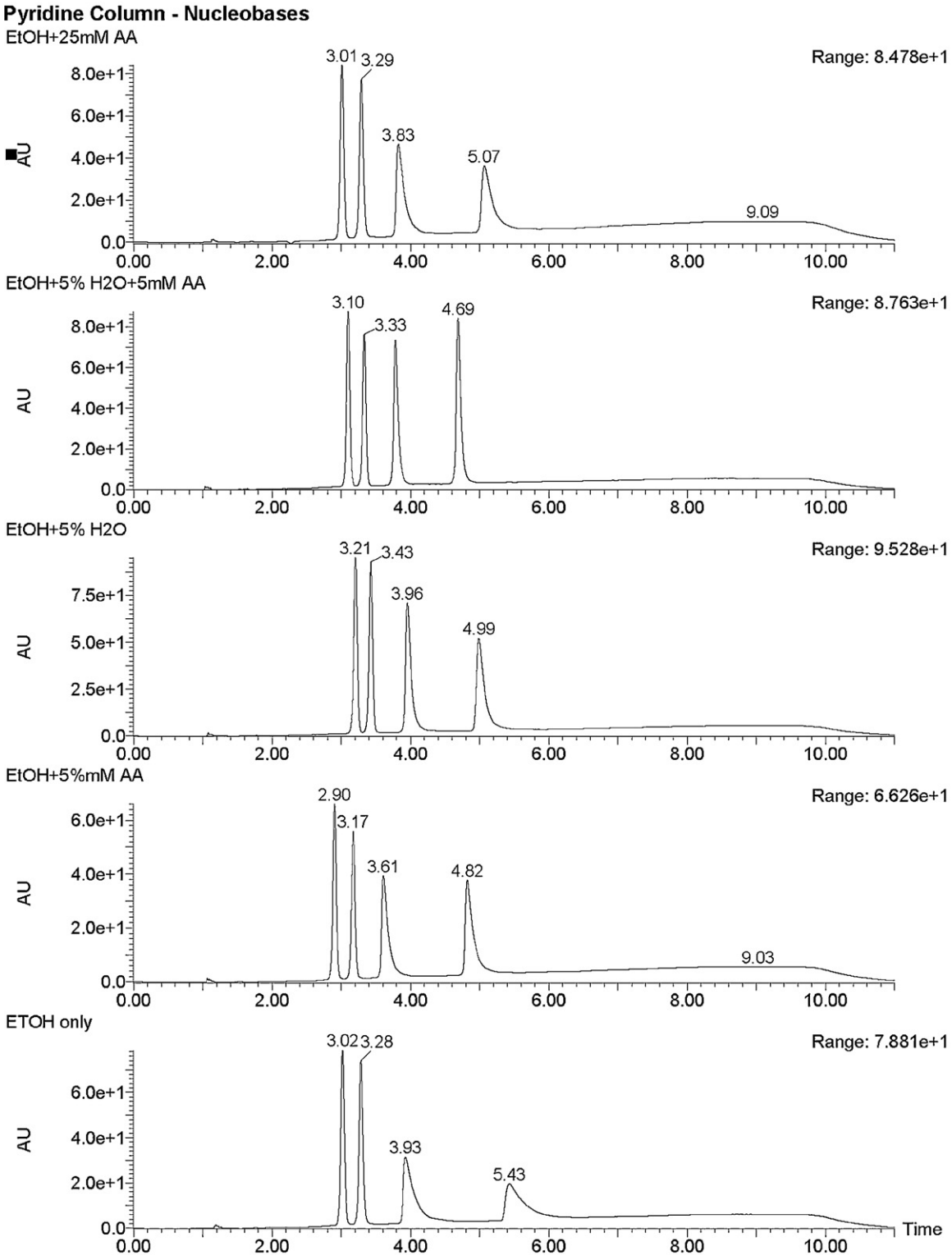

Fig. 1. Comparison of gradient separation of four component nucleobase mixture on 2-ethyl pyridine column (250 mm × 4.6 mm, d_p 5 μm, 60 Å) obtained from Princeton Chromatography with water and ammonium acetate (AA) additives and ethanol modifier. Carbon dioxide pressure and temperature were 200 atm and 40 °C. Flow rate = 3 mL/min. Order of elution: thymine, uracil, adenine, cytosine.
Ref. [11].

Isopropanol (IPA) has been suggested to offer unique properties in this regard based upon the fact the IPA has been demonstrated as an effective additive for preventing phase separation in water-containing gasoline. In pcSFC these workers discovered that IPA can allow five times more mobile phase water content than methanol. For test separations of polar analytes, the methanol system easily eluted compounds from a polymeric non-polar PRP-1 column but failed to elute the most polar analytes from a polar silica gel or diol column. For example, even 30% methanol in SC-CO_2 was inefficient to successfully elute tryptophan from the PRP-1 column.

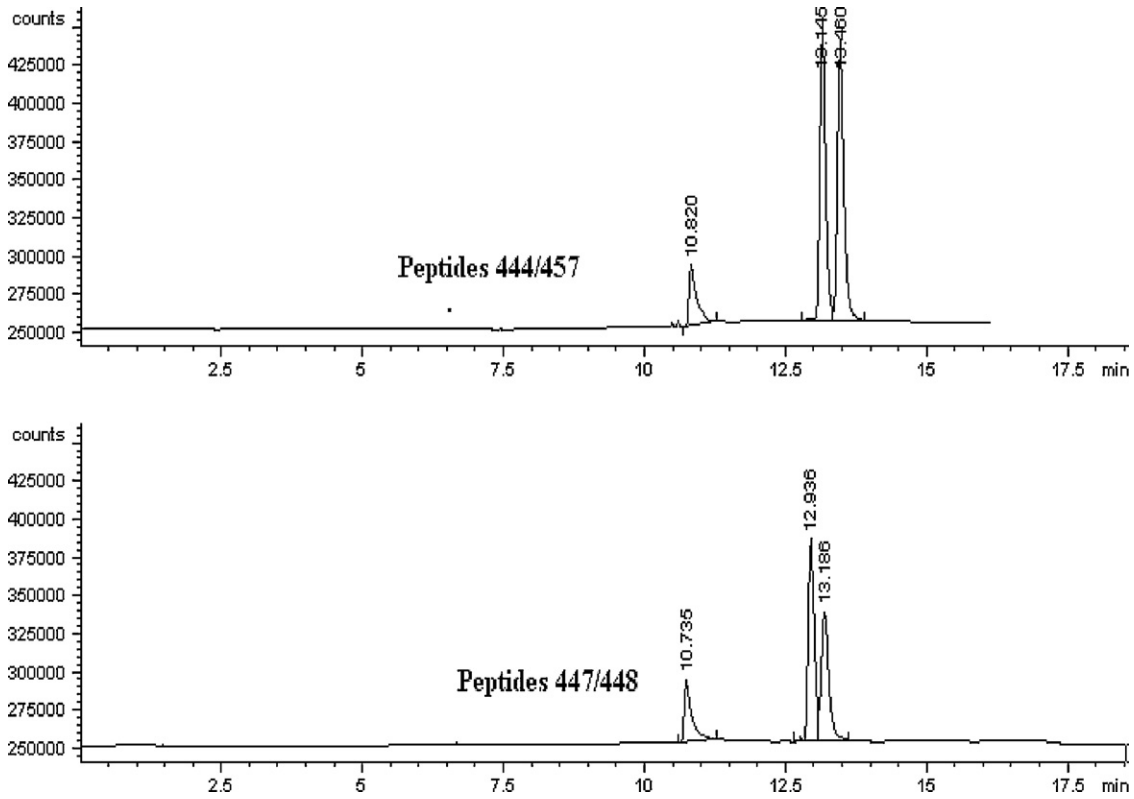

Fig. 2. Gradient SFC/ELSD separations of two isomeric peptide pairs (444/457, 1125.4 Da and 447/448, 1173.5 Da) on bare silica or diol-bonded silica from Princeton Chromatography (250 mm × 4/6 mm, d_p 5 μm) with 5% water and 0.2% TFA in methanol as mobile phase additives. Back pressure regulator was set at 120 bar and temperature was 60 °C. Flow rate = 2 mL/min.
Ref. [34].

Subsequently, as water was increasingly introduced into the methanol modifier the mobile phase polarity improved and tryptophan was ultimately eluted with a relatively decent peak shape when using 30% of a 9:1 methanol/water modifier in SC-CO_2. Similarly, tartaric acid and glutamic acid were found to readily elute with good peak shape under the same conditions. When more water was introduced into the methanol, peak shape for all analytes eroded. The same mobile phase was explored on a more retentive silica gel and diol columns. It was found that a greater percentage of the 9:1 methanol/water modifier was required for analyte elution. Some analytes only appeared when 50–60% was present. By comparison, IPA systems could elute all of the analytes from each of the columns. Of note, as the aqueous content of the IPA/water composition was steadily increased beyond 9:1, good analyte peak shape was obtained without any observation of phase separation on both the polar and non-polar stationary phases. It was concluded that IPA can increase the water capacity of SC-CO_2 and facilitate the analysis of polar hydrophilic solutes by pcSFC.

Although not pcSFC, it is worth noting that Thurbide et al. have explored the blending of CO_2 with subcritical water as a mobile phase in subcritical water chromatography (SWC) of non-polar analytes [32]. Since the polarity of water is reduced with increasing temperature, this property is used in SWC to create an isocratic mobile phase with tunable elutropic strength in reversed phase separations. Unfortunately, thermal stability of the stationary phase dictates the upper temperature limit and therefore also the minimum available mobile phase polarity. As a result SWC is often not very effective at eluting non-polar analytes. When CO_2 is blended into subcritical water, a considerable reduction in mobile phase polarity results and improves such separations. For example, in conventional SWC 1-octanol is not observed to elute from a PRP-1 column after several hours at the maximum column temperature of 200 °C. In contrast, when CO_2 is present at 180 atm in the mobile phase, 1-octanol elutes with good peak shape in less than 4 min at only 100 °C. The method was reported to extend the range of non-polar analytes amenable to SWC while maintaining the beneficial conventional SWC features of flame ionization detection and environmental compatibility.

5.3. Isomeric peptides

It should be noted that the presence of water as an additive does not always ensure the separation of choice. The example noted here concerns the separation of linear, isomeric, water soluble, ionic, decapeptides which are either capped or uncapped. The isomeric peptide pairs were of identical molecular mass (~1200 Da), composition, and charge that differed only in amino acid sequence [33]. Trifluoroacetic acid (TFA) as the primary additive in methanol proved to be the most successful modifier for elution of the two isomeric, capped peptide pairs. A variety of silica-based basic stationary phases such as 3-aminopropyl and 2-ethylpyridine were successfully employed to separate each of the isomeric pairs in the absence of water into their two components. The decapeptide pairs in this case were end-capped wherein the C-terminus was an amide group and the N-terminus was an acetyl group. The lysine side-group was protonated with trifluoroacetate as the counter ion.

The uncapped isomeric peptide pairs gave surprising results. In this study, the C and N terminus nor the side lysine group were capped. The side-group however was again protonated. TFA in conjunction with methanol as the modifier proved again to be successful for the elution (this time) of each of the carboxyl-terminated/amine-terminated peptide pairs. What was unique in this study was that the resolution of the isomeric unprotected peptide pairs was only uniquely achieved when water was

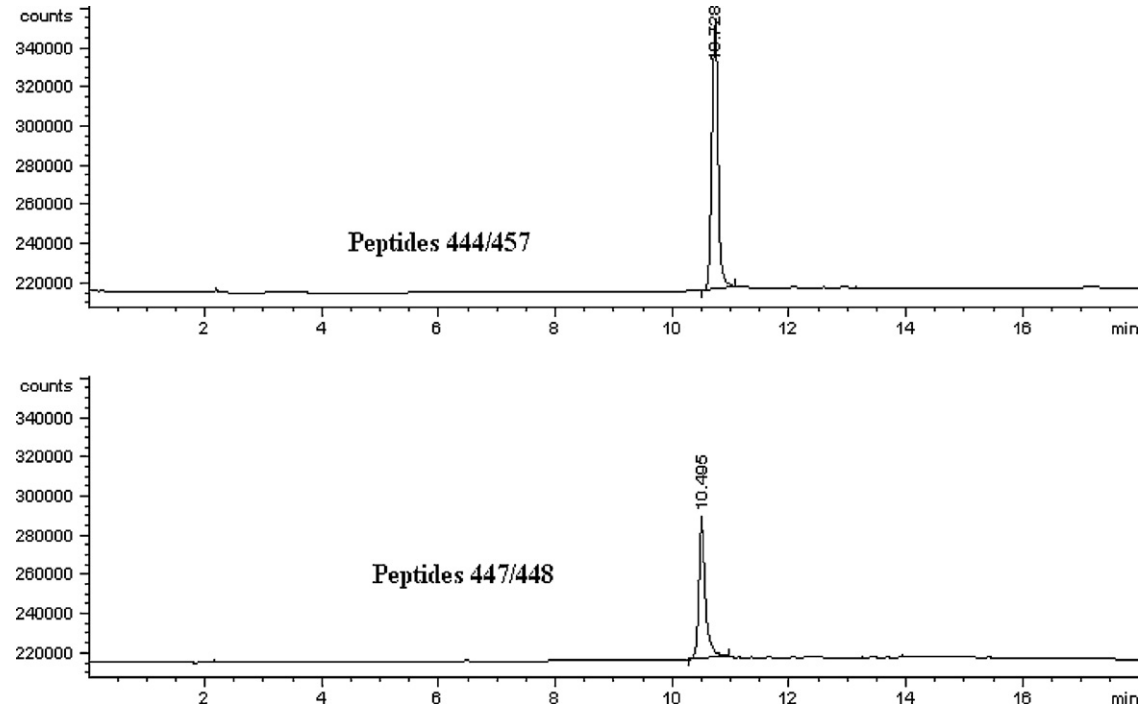

Fig. 3. Gradient SFC/ELSD separations of two isomeric peptide pairs (444/457, 1125.4 Da and 447/448, 1173.5 Da) on diol-bonded silica or bare silica from Princeton Chromatography with only 0.2% TFA as the mobile phase additive. See Fig. 2 caption for additional chromatographic details.
Ref. [34].

incorporated into the gradient mobile phase and either bare silica or diol-bonded silica was used as the stationary phase [34]. The SFC chromatographic trace which described the separation of each isomeric peptide pair with evaporative light scattering detection (SFC-ELSD) is shown in Fig. 2. The incorporation of 5% water into the mixed mobile phase coupled with bare silica as the stationary phase (e.g. Princeton Chromatography) afforded nearly baseline separation of each of the two isomeric peptide pairs. Confirmation of this separation was obtained by SFC-MS employing bare silica. The desired resolution was not observed with ELSD detection when water was removed from or reduced to 1% in the mobile phase, Fig. 3. Similar results were observed with 5% water and TFA when a diol-bonded silica stationary phase was substituted for the bare silica. In contrast, separation of the isomeric, uncapped pair was not achieved when 2-ethylpyridine with TFA and 5% water were used as mobile phase additives.

The successful employment of neutral water and TFA as primary and secondary mobile phase additives suggested two very different roles for each substance. TFA no doubt promoted protonation and ion pair formation, while water with bare silica strongly

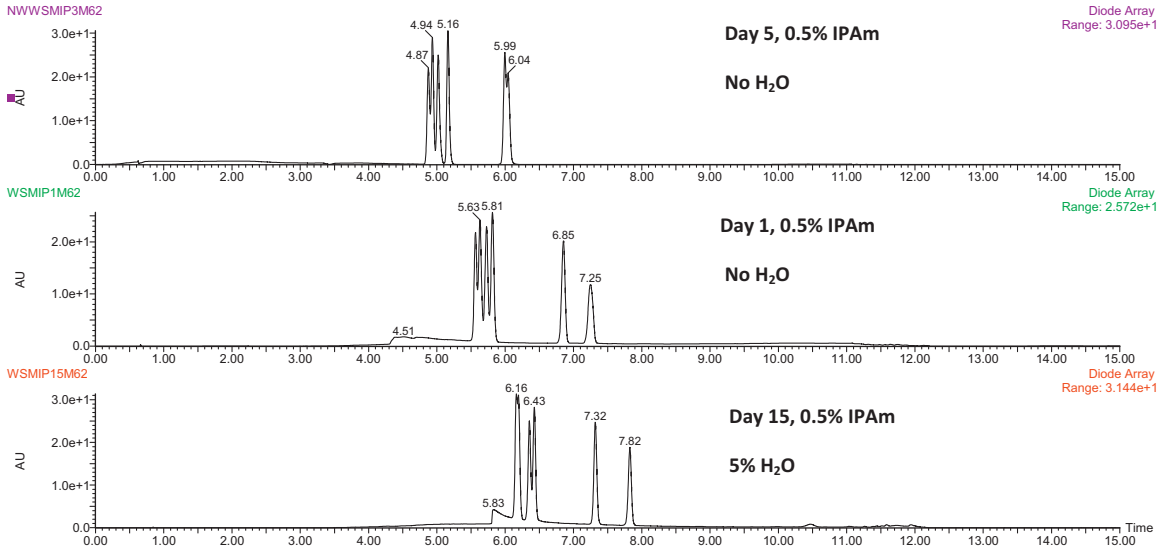

Fig. 4. Selected chromatograms for bare silica column (Waters Corp. Viridis SFC, 150 mm × 4.6 mm, d_p 5 μm) separation of basic mixture. The traces compare the separation with water in the mobile phase (lowest SFC) and with water absent from the mobile phase (top two SFC's). IPAm = iso-propylamine, flow rate = 4 mL/min. The order of elution was sulfamethazole, sulfamethoxine, sulfamerazine, sulfanilamide, sulfamethoxazole, and sulfaguanidine.
Ref. [36].

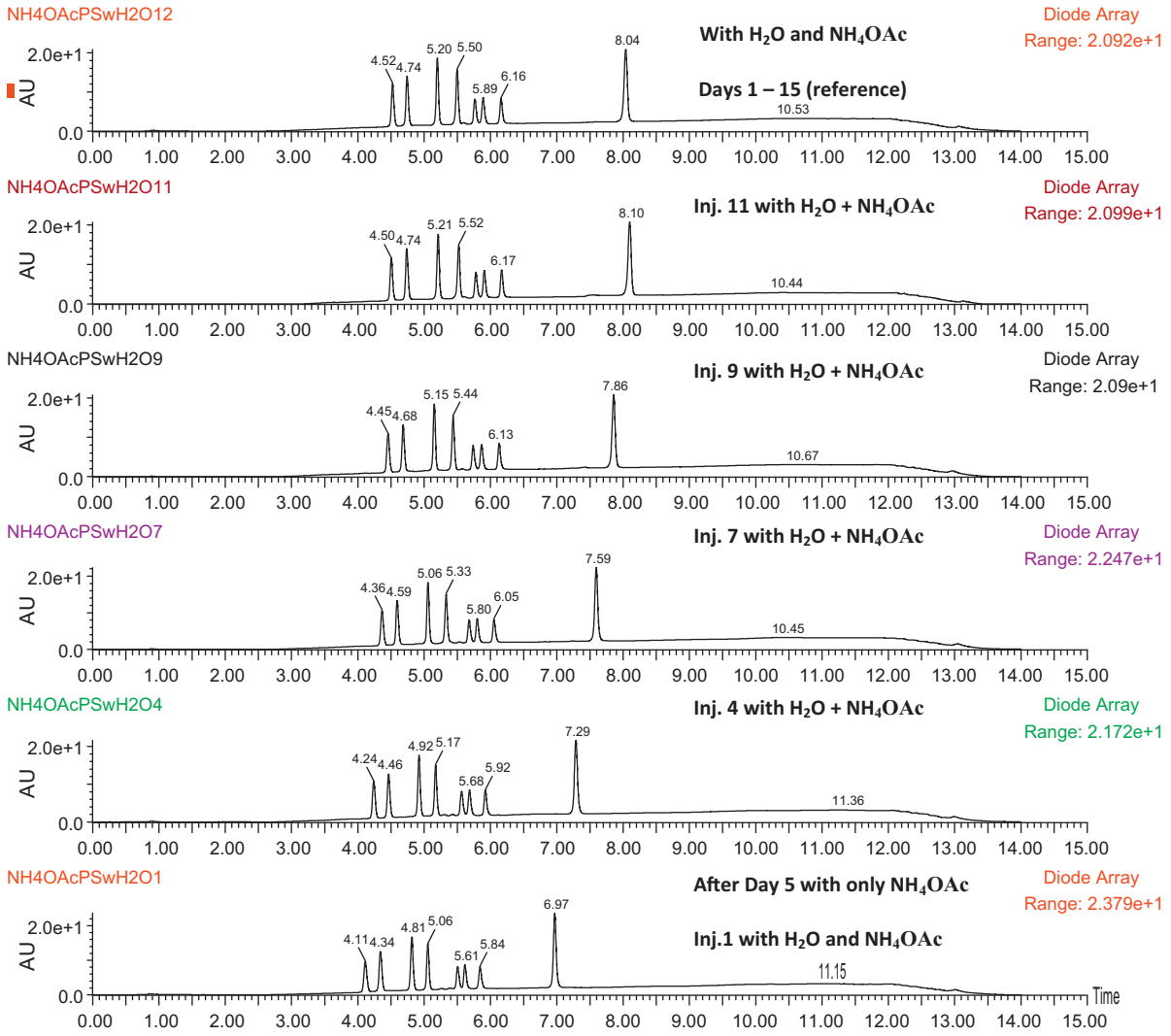

Fig. 5. Elution ofneutral mixture. Effect of adding 5% water and 10 mM ammonium acetate to the mobile phase after a five day period where no water was incorporated into the mobile phase. See Fig. 4 caption for chromatographic details. The elution order was caffeine, theophyline, thymine, uracil, cortisone, prednisone, hydrocortisone, and cytosine.
Ref. [36].

suggested a HILIC-like retention mechanism wherein analytes partition between solvated stationary phase and water in the mobile phase. HILIC-SFC may afford numerous advantages over its liquid chromatographic counterpart in that (a) lower percentages of water may be required which would lead to enhanced MS sensitivity, (b) very polar and ionic analytes can be separated, and (c) mixtures of hydrophobic and hydrophilic analytes may be retained [35]. Clearly more investigation of water promoted pcSFC separation of water soluble peptides can be justified.

5.4. Polar model analytes

The use of water as a mobile phase additive in pcSFC with a bare silica stationary phase and a gradient of CO_2/MeOH mobile phase have been recently studied [36]. Water, and in some cases, a secondary additive was mixed with the MeOH prior to gradient delivery. The primary focus was to (a) observe changes in selectivity when water is added to the mobile phase, (b) assess reproducibility of retention when water is included in SFC mobile phases, and (c) observe if the use of water as an additive permanently alters the stationary phase. Virgin packed bare silica columns from two vendors (Princeton Chromatography and Waters Corp.) donated multiple columns from their stock for separation of three test mixtures with four additive combinations. All columns were evaluated on day 1, 3, 5, 10, 15 during a 15-day schedule using the same SFC instrument, temperature, mobile phase composition, and mobile phase gradient schedule. Each column was re-equilibrated and stored between runs following the same identical procedure. At the outset, it was clear with each of the four additives that 5% water does not render the bare silica columns useless. Rather, water enhanced the separation of each of the three polarity mixtures. For example, bare silica columns from both vendors were evaluated for separation of six basic analytes with a mobile phase additive mixture composed of 0.5%IPAm + 5%H_2O in MeOH as the modifier. The elution order was 1-Sulfamethazole, 2-Sulfamethoxine, 3-Sulfamerazine, 4-Sulfanilamide, 5-Sulfamethoxazole, and 6-Sulfaguanidine. Fig. 4 shows the pcSFC/UV separation on day 15 of the mixture. Baseline resolution of compounds 5 and 6 was readily achieved as long as water was in the mobile phase; whereas sulfamethazole and sulfamethoxine were only partially resolved in the mixture. A similar situation was observed with sulfamerazine and sulfanilamide which nearly co-eluted on each of the 5-day duplicate injections during the 15-day evaluation period.

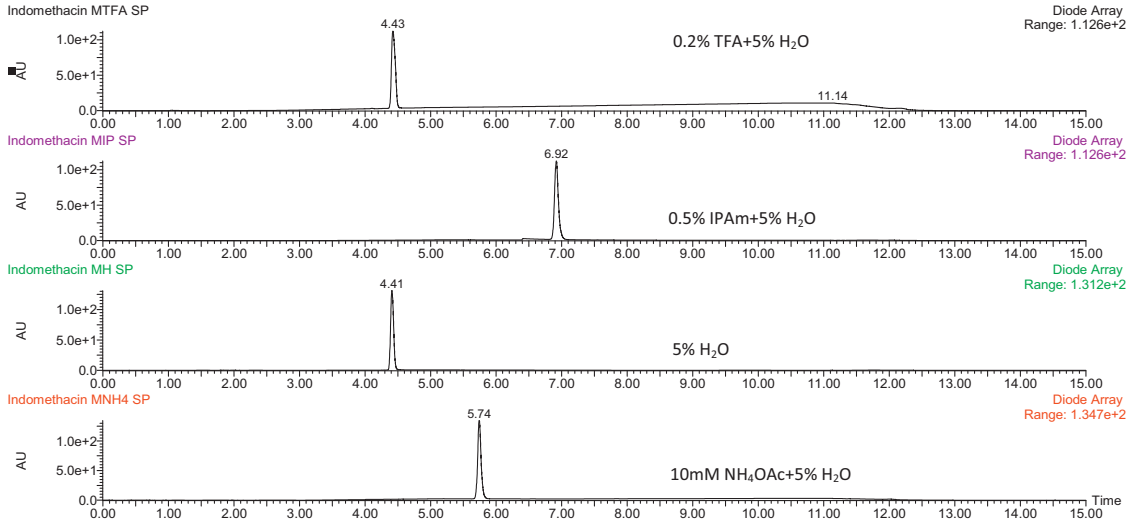

Fig. 6. Indomethacin eluted from bare silica column using 4 different additives (5% water, 0.2% ttrifluoroacetic acid, 0.5% iso-propylamine, and 10 mM ammonium acetate) via gradient SFC/UV separation. See Fig. 4 caption for additional chromatographic details.
Ref. [36].

The study next addressed the issue of bare silica chromatographic integrity in the absence of water in the mobile phase. The columns used were the same ones as used previously for water in the mobile phase. Duplicate injections were made after washing with 50/50 MeOH/CO_2 for 10 min and 100% CO_2 for 5 min followed by storing the column for an indefinite period. Each mixture composition and analyte concentration were the same as the study containing water. The gradient elution schedule was also identical to the original study. The stability of silica columns was investigated using only methanol-modified CO_2 over an additional 5-day period. The chromatographic behavior was very similar from day 1 to day 5. Fig. 4 shows the SFC/UV trace for separation of the mixture of the same six basic compounds on day 1 and day 5 of the new evaluation period.

Water as the only additive in the modifier yielded much greater selectivity for the elution of the last two compounds rather than the initial two compounds. For example, sulfamethoxazole and sulfaguanidine are baseline resolved with water, but they are only barely resolved without water. Bare silica from both vendors without water gave a much poorer separation of these two compounds. While retention times were constant with water over the 15 days, retention times of each component without water decreased with each day's injection over the 5-day period.

It was of interest to know if the incorporation of water into the mobile phase would restore the excellent selectivity and peak shapes that were initially observed. Fig. 5 describes this study with a different mixture. The composition and elution order were 1-Caffeine, 2-Theophyline, 3-Thymine, 4-Uracil, 5-Cortisone, 6-Prednisone, 7-Hydrocortisone, and 8-Cytosine. The top trace (with water, day 15) is the reference chromatogram for the mixture with water and ammonium acetate as the additives. The bottom trace (now with water re-introduced) is the result of the first injection after the study with no water in the mobile phase. Peaks are again well resolved, but peak retention times are the same as with the "no water" results. The intermediate traces shown which continue the experiment are repeats of the same injection with water in the mobile phase. Peak shapes are unchanged but retention time drifts to longer times consistent with the initial result (top trace) obtained with the bare silica and water in the mobile phase. While removal of water alters selectivity for specific analytes and retention time for all analytes in the neutral mixture, it does not destroy the chromatographic integrity of bare silica. Furthermore, re-institution of water into the mobile phase restored the excellent chromatographic behavior of bare silica for the separation of highly polar analytes.

A HILIC-like retention mechanism is envisioned wherein the silica is solvated by the more polar component (i.e. water) in the mobile phase. Analyte partitioning is hypothesized to take place between the more dense adsorbed water component and the less dense water component of the mobile phase. The reproducibility of each separation over the 15-day period regardless of the additive when water was introduced into the modifier was striking. The rapid column re-equilibration with bare silica from both vendors after gradient elution was 1 min which is far superior to what is thought to be observed with conventional normal phase liquid chromatography.

5.5. Drug molecules

The elution behavior of ten different drug molecules (Thiamphenicol, Indomethacin, Warfarin, Carbamazepin, Acetazolamide, Fenofibrate, Haloperidol, Omeprazole, Pimozide, and Niflumic Acid) using three different additive mixtures and water alone from bare silica columns was investigated [36]. Comparable SFC/UV traces with bare silica for all ten analytes using the various additives were observed. All ten molecules even though they possessed quite different chemistries surprisingly eluted as sharp peaks from bare silica when either 0.5%IPAm + 5%water or 10 mM NH_4OAc + 5%water were used as additive mixtures. Retention times for the ten compounds, however, differed considerably with these two additive combinations. Either of these two separation conditions may indeed qualify as generic mobile phases for achiral chromatography. On the other hand, haloperidol, pimozide, and omeprazole eluted with tailing and distorted peak shape when 0.2% TFA + 5% water and just 5% H_2O were used with MeOH (Fig. 6); while the other seven molecules even yielded sharp peaks with these two other additives (Fig. 7). In other words, 100% of the randomly selected drugs yielded sharp peaks and short retention times with two of the aqueous mobile phases (IPAm and AA); while 70% of the drug molecules produced sharp peaks and short retention times with aqueous TFA and water alone.

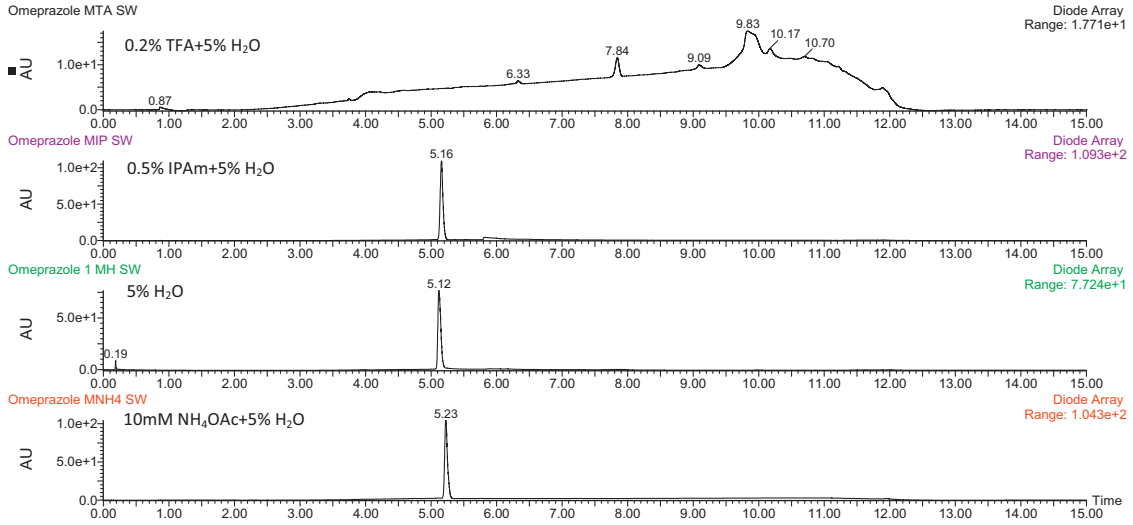

Fig. 7. Omeprazole eluted from bare silica column using 4 different additives via gradient SFC/UV separation. See Fig. 6 caption for additional chromatographic details. Ref. [36].

6. Conclusions

With faster separation, higher sample throughput, less organic solvent usage, and normal phase mode purification, pcSFC (or "carbon dioxide-based HPLC) has become a viable option for the separation scientist in such diverse areas as pharmaceuticals, foods, fragrances, natural products, polymers, metabolites, energy, propellants. Its scalability for large dimensional separations, its applicability for achiral separations, its ability for separation of water soluble analytes, and its feasibility for coupling columns of varied chemistries is only beginning to be appreciated. The fields in which pcSFC could be applied become larger every month. The use of additives to dramatically extend the range of solute polarity amenable to CO_2 base pcSFC was predicted as early as 1991 [9]. At that time additives were predicted to have multiple functions. Lesellier has noted that the retention behavior in pcSFC first depends on the stationary phase nature [8]. The adsorption of mobile phase components on the stationary phase causing a modification of its surface thus becomes highly significant. One role of additives that has not received much discussion concerns changing the polarity of the stationary phase. As evidenced by the studies described in this review, water may offer much potential as an additive in that it could (a) enhance the solvating power of the mobile phase, (b) introduce HILIC-like analyte partitioning, (c) simplify preparative separations, and (d) be more mass spectrometrically compatible. A greater fundamental understanding of stationary phase modification by mobile phase components appears to be well justified

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