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Supercritical fluid chromatographic resolution of water soluble isomeric carboxyl/amine terminated peptides facilitated via mobile phase water and ion pair formation

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ABSTRACT

Both analytical scale and preparative scale packed column supercritical fluid chromatography (SFC) have found widespread applicability for chiral separations of multiple polar pharmaceutical candidates. However, SFC is rapidly becoming an achiral technique. More specifically, ion pair SFC is finding greater utility for separation of ionic analytes such as amine salts and organic sulfonates. The key to this success is, in part, the incorporation of additives such as trifluoroacetic acid and ammonium acetate into the mobile phase in association with a wide variety of both bonded silica stationary phases and high purity bare silica. Ion pairing SFC coupled with evaporative light scattering detection and mass spectrometric detection is presented here for the separation of water soluble, uncapped, isomeric peptide pairs that differ in amino acid arrangement. The separation is best achieved on either diol-bonded silica or bare silica with 1–5% (w/w) water as a significant ingredient in the mobile phase. Nitrogenous stationary phases such as 2-ethylpyridine, which had been very successful for the separation of capped peptides failed to yield the desired separation regardless of the mobile phase composition. A HILIC type retention mechanism is postulated for the separation of both isomeric uncapped peptide pairs.

1. Introduction

The use of mobile phase additives has been quite successful for separation of polar analytes via packed column supercritical fluid chromatography (pcSFC) [1,2]. The additives are generally added directly to the modifier and then pumped as a single binary fluid thereby augmenting the solvating power and flow of pressurized carbon dioxide. Additives are thought to perform at least five different functions [3]. These include: (a) cover active sites on the solid support, (b) change the polarity of the stationary phase, (c) suppress ionization of the analyte, (d) enhance ion pair formation with the analyte, and (e) raise the solvating power of the mobile phase. Of these five functions, modification of the stationary phase may be the most critical function. Maximum adsorption of CO_2 has been found to occur near its critical temperature and critical pressure [4]. Parcher et al. have suggested that a thick film of CO_2 may be coating the stationary phase and acting as a part of it. Modifiers have been shown to strongly adsorb to the stationary phase [5], and additives such as organic acids and bases, inorganic salts, and water are expected to do likewise.

Low to moderately polar stationary phases such as octyl and cyanopropyl were observed to adsorb only small amounts (0.4–0.6% of a monolayer) of modifier and additive. Under the same conditions, sulfonic acid and diol columns adsorbed much larger amounts of modifier/additive creating surface coverage up to 21% of a monolayer [6]. Basic, acidic, and salt additives have proven most popular, but as this report will attest, neutral water offers some significant advantages. Such behavior is not unique to SFC since it has been theorized and now demonstrated that a water-rich layer is associated with the silica surface in hydrophilic interaction chromatography (HILIC) [7].

Applications of ion-pairing SFC have been demonstrated in the past [8,9]. Pinkston et al. brought new thinking to this area when they showed that low levels of volatile ammonium salts as mobile phase additives allowed the elution of polar and even ionic organic materials from a Deltabond cyanopropyl-bonded silica column [10]. However, it was not clear from this study whether the salt ion paired with the analyte or if the salt interacted with the stationary phase. In a more recent study, the effect of various ammonium salts on the elution of *anionic* compounds such as the sodium salts of 4-dodecylbenzene sulfonate and 4-octylbenzene sulfonate has been shown [11]. The successful elution of *cationic* species (i.e. $NR_4^+X^-$), from a Deltabond cyanopropyl-bonded silica column was achieved by adding sodium alkylsulfonate to the methanol-modified carbon

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dioxide-based mobile phase. An ion pairing interaction between the positively charged amine cation and the anionic alkylsulfonate ion was proposed [12].

In some cases, conversion of a neutral analyte into an ion actually enhances SFC separation by favoring an ion-pairing mechanism. Stringham has reported that incorporation of a strong acid in both the sample diluent and mobile phase modifier gives a dramatic improvement in the separation of chiral basic compounds [13].

The re-discovery that the use of water as a neutral additive can lead to striking separations with bare silica has given new emphasis to the area of packed column SFC. Subcritical fluid chromatography of monosaccharides and polyols using bare silica and trimethylsilyl columns with a mobile phase composed of CO_2–methanol–water was reported nearly 15 years ago [14]. By adjusting the column temperature to 60 °C and the flow rate to 5 mL/min, a complete separation of eight underivatized monosaccharides was obtained in less than 10 min. Higher efficiencies and resolution were obtained when both water and triethylamine were added to methanol as the polar modifier.

Water, of course, is impractical as a direct modifier due to its very low solubility (~0.1%) in supercritical CO_2, but water blended as a tertiary component into alcohol modified supercritical CO_2 can assist in packed column SFC separations of highly polar analytes and thereby expand the capabilities of the technique. In this regard, 2-propanol and methanol have been compared as alcohol/water modifiers of CO_2 [15]. More recently, one study has described the packed column SFC separation of nucleobases on a variety of stationary phases with methanol/water as the modifier/additive [16]. In another study, numerous polar analytes and drug-like molecules have been shown to yield sharp peaks when eluted from bare silica with a mixture of methanol–water–CO_2 [17]. The removal of water from the modifier resulted in a decrease in selectivity and an increase in retention factor, however, the sharp peak shape was still achieved when water was removed from the mobile phase. The excellent chromatography could be restored via incorporation of water into the mobile phase. Another interesting feature of water as an additive is its compatibility with preparative SFC.

Successful pcSFC of polypeptides has employed a variety of mobile phase additives [18]. Blackwell and Stringham eluted peptides such as bradykinine and oxytocin from a divinylbenzene polymeric (i.e. 500 Å) column using heptadecafluorooctanesulfonic acid as an additive in methanol with CO_2 [19]. The separation of polypeptide salts containing a variety of acidic and basic residues was achieved via pcSFC–MS when trifluoroacetic acid (TFA) was used as the additive in a CO_2/methanol mobile phase on a 2-ethylpyridine–silica column [20].

With the discovery that ionic peptides of moderately high molecular mass can be eluted via pcSFC by incorporation of selected mobile phase additives, our interest has turned to the separation of polypeptide mixtures. A study was recently reported wherein the separation of two pairs of linear, water soluble, 12mer peptides of identical molecular mass (~1200 Da), composition, and charge that differ only in amino acid sequence was reported [21]. TFA as the additive in methanol proved to be the most successful modifier for elution of the two isomeric peptide pairs. A variety of silica-based basic stationary phases such as 3-aminopropyl and 2-ethylpyridine successfully eluted the isomeric peptide pairs. 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 but not capped.

We would now like to report the separation of water soluble, decapeptide pairs of identical mass, composition, and charge that differ only in amino acid sequence. In this study, the C and N terminus nor the side lysine group have been capped. The side-group however was again protonated. As in our previous work referenced above, TFA in conjunction with methanol as the modifier proved to be successful for the elution of each of the carboxyl-terminated/amine-terminated peptide pairs via IPSFC. What is unique in this study is that the resolution of the isomeric unprotected peptide pairs was uniquely achieved when water was incorporated into the gradient mobile phase and either bare silica or diol-bonded silica was used as the stationary phase [22].

2. Experimental

2.1. Peptides and sample preparation

Uncapped peptides containing both an amine and carboxyl terminus were synthesized by CPC Scientific Inc. (San Jose, CA) for this research. The linear isomeric peptides exhibited high water solubility (e.g. 1 mg/mL). One pair of peptides (CI-05-00457 and CI-05-00444) exhibited a molecular mass of 1125.4 Da and the other pair of isomeric peptides (CI-05-00447 and CI-05-00448) had a molecular mass of 1173.5 Da. Each peptide pair had the same composition and exhibited minimal variation in the amino acid sequence. The former pair (1125.4) differed only in the placement of one of the glycines (G) and the lone valine (V) in the chain. The latter peptide pair (1173.5) differed only in the placement of one of the glycines (G) and the lone phenylalanine (F). Each peptide chain contained two lysine units with one nitrogen atom in the linear chain and the second nitrogen appearing as a dangling amino function that was no doubt positively charged in the acidic CO_2-containing mobile phase. The peptides supplied by CPC Scientific Inc. were in fact stated to be salts of trifluoroacetate, which served as the negative counterion. The amino acid sequence along with the quoted molecular mass and injected sample solution concentration for each of the amine/carboxyl terminated, cationic peptide chains are given in Table 1. Samples for chromatographic injections on the SFC–MS were prepared by pipetting a 50:50 mixture of each peptide pair, for example 0.5 mL of CI-05-00444 + 0.5 mL of CI-05-00457. Samples for chromatographic injections on the SFC–ELSD were prepared in a similar fashion although the total volumes were smaller.

2.2. Instrumentation and chromatographic conditions

All experiments performed at Virginia Tech were done using a Waters Corp. (Milford, MA) SFC equipped with a high pressure pump, evaporative light scattering detector (Alltech ELSD), autosampler, oven heater set to 60 °C, and back pressure regulator set at 140 bar. All SFC analyses were performed via gradient elution. All experiments performed at Pfizer were done using a Waters Analytical method station II with a ZQ mass spectrometer (Milford, MA). The back pressure regulator was set at 120 bar and the oven temperature was set at 60 °C. The percent additive in the methanol modifier is given in the figure captions. The flow rate was 2 mL/min and the linear gradient schedule for this study is given in Table 2. For ELSD measurements, the outlet flow was split post-back pressure restrictor (BPR) such that only 1/4 of the flow passed through the ELSD. The ELSD drift tube temperature was set to 105 °C and the nitrogen flow was set to approximately 3.5 L/min. The split ratio was adjusted with a fused silica restrictor. Sample injection volume was 5 μL and the concentration of the injected solution was approximately 0.5 μg/μL/peptide isomer. In addition to bare silica, a number of polar and non-polar stationary phases were examined as candidates for both efficient elution of each peptide and near baseline resolution of the isomeric carboxyl/amine terminated peptide salts under supercritical conditions. A list of all columns extensively investigated in this study can be found in Table 3.

Table 1
Peptide sequence and concentration of injected solution. The amino acid residues that are interchanged are shown in bold. Peptides were synthesized September 15, 2009. Purity of peptides as determined by HPLC ranged from 83.4% to 87.0%. Stated water solubility: 1 mg/mL. Counter ion: trifluoroacetate.

Lot number	Uncapped peptides	Molecular mass (Da)	Pfizer conc. (mg/mL)	VT conc. (μg/μL)
CI-05-00444	G**V**LGLALG**G**LKK	1125.4	0.4	0.5
CI-05-00457	G**G**LGLALG**V**LKK	1125.4	0.4	0.5
CI-05-00447	G**F**LGLALG**G**LKK	1173.5	0.4	0.5
CI-05-00448	G**G**LGLALG**F**LKK	1173.5	0.4	0.5

Table 2
pcSFC gradient schedule at each location.

Time (min)	CO_2 (%)	Modifier (%)
0	95	5
1.0	95	5
8.5	50	50
13.5	50	50
14.0	95	5
17.5	95	5

Table 3
Columns tested in this study.

Column	Dimensions (mm)	Particle size (μm)	Column vendor
2-Ethyl pyridine	4.6 × 250	5	Princeton
Silica	4.6 × 250	5	Princeton
Diol	4.6 × 250	5	Princeton
Luna C18	4.6 × 250	5	Phenomenex

3. Results and discussion

At the outset of the work with uncapped, linear isomeric peptides it was thought that the conditions for separation would be similar to what proved successful with the analogous capped peptides [21]. In this case, resolution of the capped isomeric peptide pairs was achieved with a nitrogenous, silica-based stationary phase such as 3-aminopropyl or 2-ethylpyridine with TFA additive in methanol-modified CO_2. Fig. 1 reveals our finding with one of the unprotected peptide binary mixtures under these conditions with selective ion mass spectrometric detection. Resolution of the isomeric pair via gradient elution with methanol on 2-ethylpyridine was not achieved with a variety of modifier (MeOH)/additive combinations: 0.1% TFA/methanol, 0.1% TFA/methanol/10% water or 0.1% TFA/0.1% IPAm/methanol/10% water, where IPAm = isopropylamine. Each of the two component peptide mixtures yielded a single sharp peak instead of two peaks with each of the three modifier/additive combinations selected.

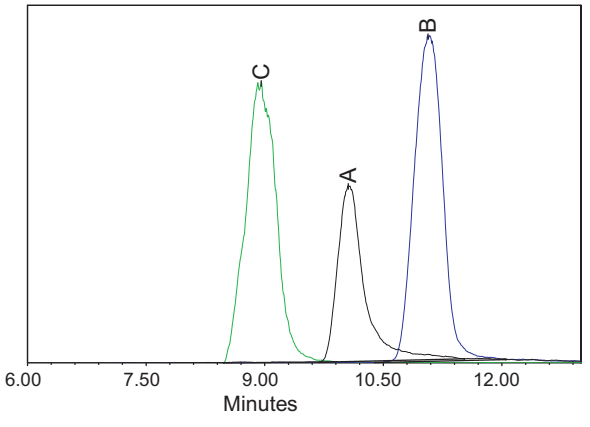

Fig. 1. Overlaid SFC/MS chromatograms of isomeric peptide pair 447/448 on 2-ethyl pyridine column. *A* = 0.2% TFA in methanol, *B* = 0.2% TFA in 90:10 methanol:water, *C* = 0.1% TFA + 0.1% IPAm in 90:10 methanol:water (for illustration purposes the scale and *x*-axis of the chromatograms have been altered).

Similar results were obtained with the 3-aminopropyl silica-based stationary phase when acidic, neutral, and basic additives were used.

A variety of hydrophobic stationary phases were screened next. Since mobile phase components are known to yield monolayers on stationary phases, it was of interest to initially know the effect of mobile phase-containing water on a silica-bonded hydrophobic stationary phase such as C18. Would water solvate the stationary phase thereby creating stagnate pools of water and thus introduce a more efficient retention mechanism based upon partitioning with water in the mobile phase rather than absorption onto the solid support for elution of the peptide mixture? The SFC/MS chromatographic trace of a four component peptide mixture when injected onto a C18 column with a gradient of CO_2 and methanol containing 0.2% TFA was unacceptable regardless of the extracted ion selected, i.e. *m*/*z* – 1126 or *m*/*z* 1174. When water was introduced into the modifier at 10%, better (but not great) chromatography was produced but only a single peak was observed for each isomeric pair. When IPAm was introduced as an additive along with TFA into the modifier/water mixture, single sharp peaks were observed for each isomeric pair, but selectivity for the two isomers was lost completely on the same C18 packed column phase, Fig. 2. In other words, water appeared to have changed the hydrophobic phase into a hydrophilic phase. Although our goal of isomer separation was not achieved, water did appear to enable better elution of the unprotected peptides. With the knowledge that the addition of water enhanced the chromatography of unprotected peptides from a hydrated hydrophobic stationary phase, our efforts turned to experiments using bare packed silica.

Unlike our previously reported separations via ion pairing SFC/MS of identical isomeric *endcapped* peptide pairs, separation of the amine-/carboxyl-terminated peptides was surprisingly achieved via bare silica with TFA and water as binary mobile phase additive components [23]. The SFC chromatographic trace, which describes the separation of each isomeric peptide pair with evaporative light scattering detection (SFC–ELSD) is shown in Fig. 3. 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

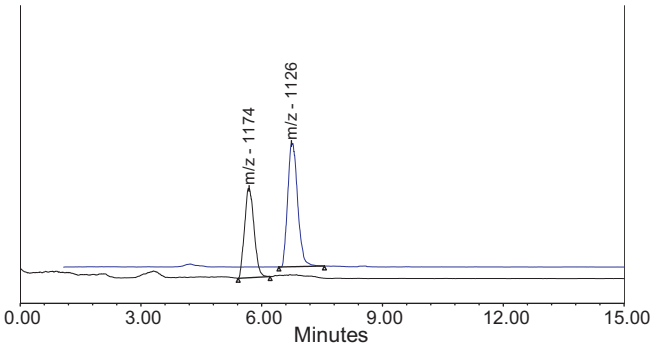

Fig. 2. SFC–MS of isomeric peptide pair on C18 with gradient of CO_2 and 0.1% TFA/0.1% IPAm in 90:10 methanol:water (for illustration purposes the scale and *x*-axis of the chromatograms have been altered).

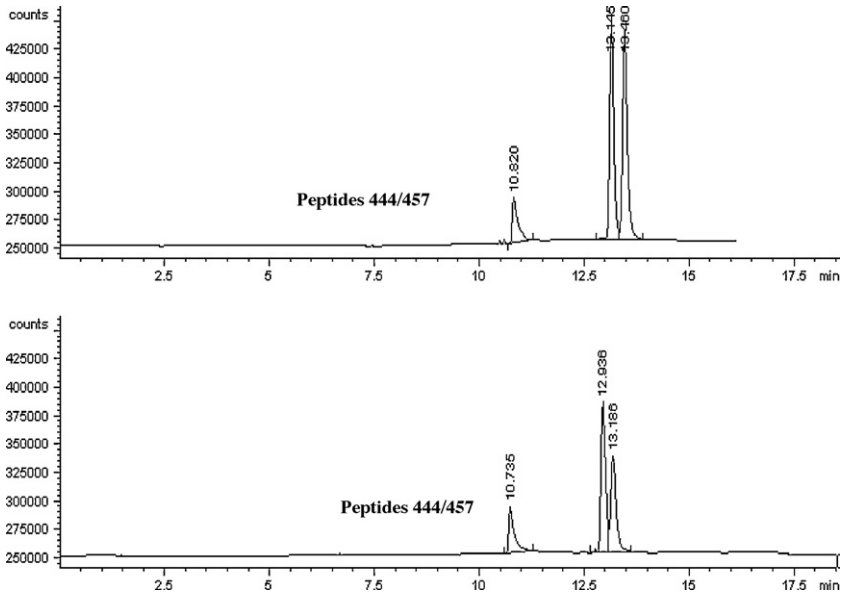

Fig. 3. Gradient SFC/ELSD separations of two isomeric peptide pairs on Princeton bare silica with 5% water and 0.2% TFA in methanol as mobile phase additives. Identification of the peptides is described in Table 1.

peptide pairs. Confirmation of this separation was obtained by SFC–MS employing bare silica as shown in Fig. 4a and b. Bare silica from Phenomenex did not produce the same resolution. The desired resolution was also not observed with ELSD when water was removed from or reduced to 1% in the mobile phase, Fig. 5. This observation was also confirmed by SFC/MS at the Pfizer laboratory. Similar results, however, were obtained with 5% water and TFA when a diol-bonded silica stationary phase was substituted for the bare silica, Fig. 6. In contrast, separation of the isomeric pair was not achieved when 2-ethylpyridine with TFA and 5% water were used as mobile phase additives. It is important to remember that separation of the *endcapped* isomeric peptide pairs was achieved in multiple fashions with a variety of nitrogenous stationary phases with equal success, but only when water was not in the mobile phase. The combination of water and bare silica for resolution of these simple model peptide pairs yielded a striking and surprising result.

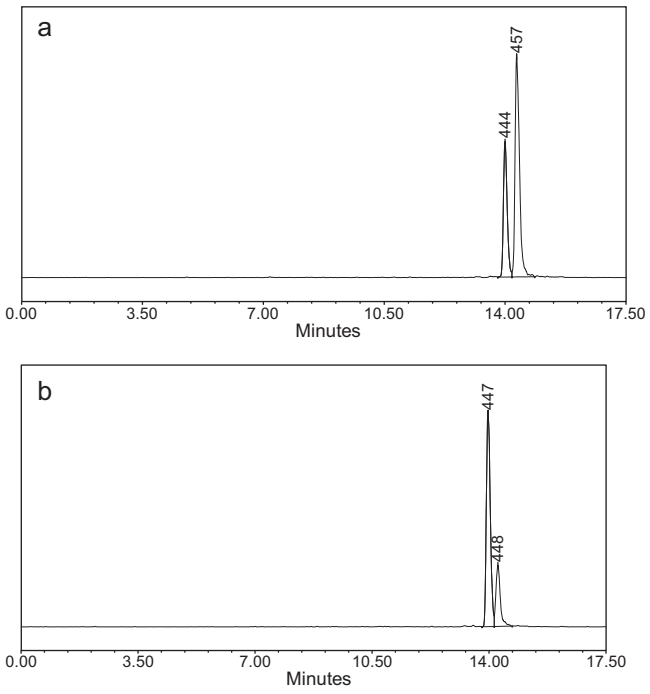

Fig. 4. (a) Gradient SFC/MS separations of two isomeric peptide pairs 444 and 457 with $m/z = 1126$ on Princeton bare silica column with 0.2% TFA in 95:5 methanol:water. (b) Gradient SFC/MS separations of isomeric peptide pairs 447 and 448 with $m/z = 1174$ on Princeton bare silica column with 0.2% TFA in 95:5 methanol:water.

The effective gradient water concentration ranged from 0.5% to 5%. At these concentrations the addition of water probably has consequences on analyte log *D* and can potentially affect retention and peak shape. Thus, the use of a single pH in this work is unlikely to produce optimal chromatographic conditions for separations. A direct measurement of the pH of water in contact with supercritical CO_2 at pressures of 70–200 atm and temperatures of 25–70 °C has been previously shown to vary from 2.80 to 2.95 [22]. This pH effect may or may not be optimal for these stationary phases; nevertheless, no pH adjustment or study was performed in the work reported here.

This study lends support to the notion of Berger and Deye over 15 years ago which stated that the greatest successful strategy for improving SFC chromatographic peak shapes of polar solutes is achieved using polar modifiers and even more polar additives with polar stationary phases [3]. The optimized chromatographic conditions noted here appear to complement the stated criteria. The dominant retention mechanism for solutes in SFC, we feel, then becomes the interaction between the solutes and the most polar entity on the chromatographic support which in our case must be water. Berger and Deye suggested that to be successful in SFC the stationary phase should be more polar than the solutes and probably more polar than a silanol site [6]. Thus, it is now assumed that a film of modifier and CO_2 is a significant, if not dominant part of the true packed column stationary phase since the film tends to further decrease the intensity of the solute–silanol interaction. Under supercritical fluid conditions, surface molar excesses of mobile phase additives may also act to change the chemical nature of the stationary phase surface thereby making it more polar than the mobile phase.

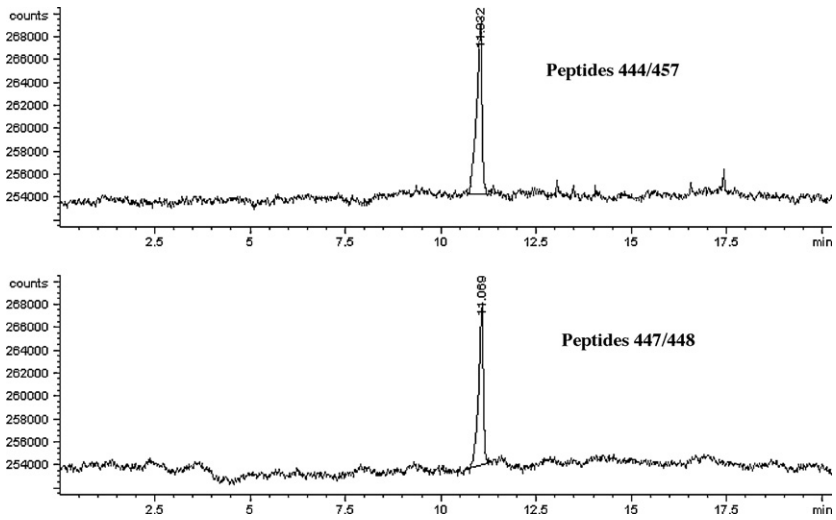

Fig. 5. Gradient SFC/ELSD separations of two isomeric peptide pairs on a bare silica column with only 0.2% TFA as the mobile phase additive. See Table 1 for identification of each peptide.

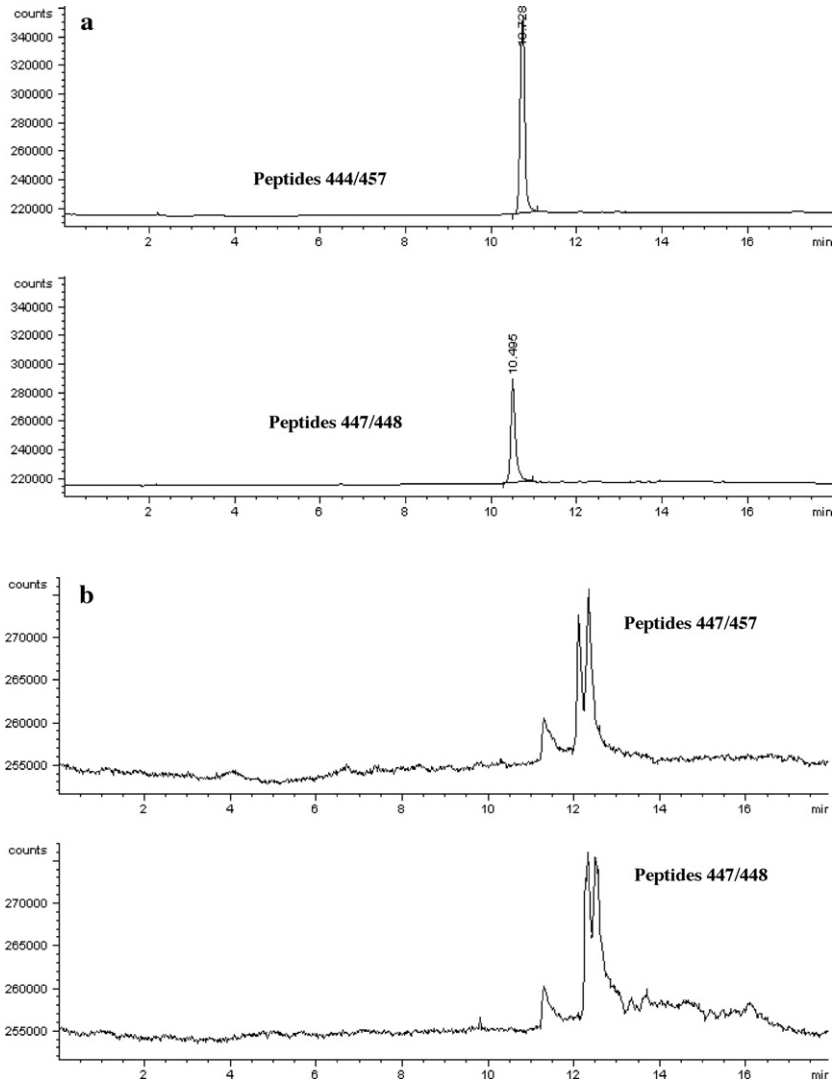

Fig. 6. (a) Gradient SFC/ELSD separations of two isomeric peptide pairs on a diol-bonded silica column with only 0.2% TFA as the mobile phase additive. See Table 1 for identification of each peptide. (b) Gradient SFC/ELSD separations of two isomeric peptide pairs on a diol-bonded silica column with 5% water and 0.2% TFA as the mobile phase additive. See Table 1 for identification of each peptide.

It is clear that polar, high molecular mass, protonated polypeptides can be chromatographed employing ion pairing SFC. Further work is encouraged in this area where hydrophobic and hydrophilic peptides of different molecular mass, charge, and amino acid sequence are studied. Evidence to date indicates that ion pairing SFC of polypeptides has a bright future. The successful employment of neutral water and TFA as mobile phase additives suggests two very different roles for each substance. TFA no doubt promotes protonation and ion pair formation, while water with bare silica strongly suggest 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 [24].

Packed column SFC has experienced tremendous growth as an analytical and preparative technique over the past 20 years. Unlike GC and HPLC, where various retention models have been extensively developed for years, SFC suffers from the lack of systematic studies on retention mechanisms and useful models for solute retention. The key to the development of achiral SFC may lie with highly acidic ionic additives in conjunction with ion pairing principles. SFC of highly polar analytes has been demonstrated on numerous occasions over the past 40 years. As more separation scientists grasp the true potential of SFC, new applications are expected with enhanced mechanistic and thermodynamic understanding.

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