Anal Bioanal Chem (2010) 396:775–781
DOI 10.1007/s00216-009-3228-4

ORIGINAL PAPER

Separation of peptides by HPLC using a surface-confined ionic liquid stationary phase

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Received: 27 July 2009 / Revised: 5 October 2009 / Accepted: 10 October 2009 / Published online: 31 October 2009

Abstract A butylimidazolium bromide surface-confined ionic liquid stationary phase was synthesized in-house. The synthesized phase was investigated for the separation of five peptides (Gly-Tyr, Val-Tyr-Val, leucine enkephalin, methionine enkephalin, and angiotensin-II). The peptides were successfully separated in less than 5 min. The effect of trifluoroacetic acid (TFA) on the separation of peptides was evaluated with results confirming that TFA was not acting as ion-pairing agent in separation of peptides on this phase.

Keywords Peptides · Retention mechanisms · Surface-confined ionic liquids · HPLC · Separations/theory

Introduction

Peptides have significant roles in biochemical function, physiological processes, molecular biology, clinical research and in the identification of proteins in proteomics [1, 2]. These important applications have led to the increased momentum for techniques that effectively separate them. The technique of choice for the separation of peptides and proteins is high-performance liquid chromatography (HPLC) [3, 4].

The challenges associated with peptide separations are due to nature of peptides themselves such as their diverse chemical nature (e.g., hydrophobic and hydrophilic sites, acidic and basic groups, and aromatic rings) and variability in size, shape, and charge. Typically, they are present as complex mixtures. Thus, the separation of peptides often requires the application of several strategies and chromatographies in their separation.

In HPLC, different chromatographic modes such as reversed-phase [5–8], ion exchange [9, 10], size exclusion [11, 12], hydrophilic interaction [13, 14], and affinity chromatography [15, 16] are predominantly used for successful separation of peptides. Depending upon the complexity of peptide mixture, a series of chromatographic techniques are sometimes employed for their effective separations.

The method of choice for over two decades in separation of peptides is reversed-phase (RP) HPLC, where stationary phases are made of hydrocarbon ligands (e.g., C-4, C-8, C-18, and phenyl phases). Recently, multimodal macrocyclic antibiotic phases and porphyrin-based phases have demonstrated utility for peptide separations [2, 17]. In RP-HPLC different gradient elution schemes with polar and non-polar solvents are used for the separation of peptides, and the primary mode of interaction of peptides with these phases is purely hydrophobic; hence, more hydrophobic peptides are more retained on the hydrophobic phases. In RP-HPLC of peptides, ion-pairing agents are commonly used as mobile phase additives.

Ion-pairing agents are selected depending upon the charge of the peptides of interest to be separated. The homologous series of volatile perfluorinated acids—trifluoroacetic acid (TFA), pentafluoropropionic acid, and heptafluorobutyric acid—proved to be excellent ion-pairing agents for RP-HPLC of peptides [18–21]. These ion-pairing agents affect the peptide retention behavior during RP-HPLC by interaction of the ions with oppositely charged functional

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groups on the peptides (e.g., basic side chains Lys, Arg, His, and free terminal α-amino group interacting with ion-pairing agent anion), causing a net reduction in the hydrophilicity of peptides; the alkyl groups of the ion-pairing ions impart hydrophobic character to the ion pair resulting in increased hydrophobic interaction with the reversed-phase sorbent.

Of all the perfluorinated acids, TFA is the most extensively used mobile phase additive due to its UV transparency, excellent solubilizing properties, volatility, availability, and high purity [22]. Generally, the concentration of acidic agents employed is 0.05–0.1% (*v*/*v*) with increasing concentration causing longer retention of peptides. However, concentrations of these ion-pairing agents must be limited because of the vulnerability of silica-based bonded phases to acid hydrolysis.

Molten salts, also known as ionic liquids, are defined as liquids which are composed solely of ions [23, 24]. Ionic liquids are typically composed of large asymmetrical organic cations coupled to inorganic anions [25, 26]. Room temperature ionic liquids (RTILs) are molten salts with melting points at or below approximately 100 °C. RTILs have a diverse range of applications [23, 25] (e.g., solvents in organic synthesis [27–29], matrices in matrix-assisted laser desorption/ionization mass spectrometry [30, 31], additives or coatings in capillary electrophoresis [32–34], and stationary phases in gas chromatography [35–40]). However, they do have limited applicability in HPLC as mobile phase additives [41]. Because of the relatively high viscosities of RTILs, their concentrations in mobile phases must be kept relatively low (1–10 mM) [42]. Immobilization of RTIL motifs onto a silica substrate does not technically constitute an ionic liquid; nevertheless, it has been shown that these phases may retain some ionic liquid-like properties (e.g., partitioning behavior) [43] and affords some unique selectivities as stationary phases in HPLC [44].

Currently, a number of research groups are utilizing similar methods to covalently attach various RTIL cations, primarily alkylimidazolium, with a variety of associated anions (e.g., chloride, bromide, iodide, and tetrafluoroborate) to silica sorbents, forming surface-confined ionic liquid stationary phases for HPLC [43, 45–51]. Changing the identity of cation and anion combination on the surface of silica during the synthesis leads to the development of numerous new surface-confined ionic liquid (SCIL) phases and the multiple retention modalities (i.e., hydrophobic, electrostatic, and hydrogen bonding) in which these new bonded phases can participate have shown utility in the separation of different classes of compounds [43, 45, 48, 51]. The reports published by various groups have primarily focused on the characterization of SCIL phases in terms of physical characteristics [25, 47, 48] and their ability to separate aromatic carboxylic acids [25], ephedrines [45], inorganic ions [47, 48], nucleotides [43], and geometric isomers [51].

The retention mechanism on these SCILs relies on complex intermolecular interactions, which are dependent upon the kind of analytes used and solvent system employed. Indeed, it was shown that despite the presence of a positively charged imidazolium ring, these phases had a phenyl-type reversed-phase chromatographic mechanism for neutral aromatics. However, published results on use of SCILs [43] for the separation of nucleotides have shown that these phases behave like strong anion exchange stationary phases, further making these phases an interesting point of study. As both modes have proven useful in the separation of peptides [6, 10], our work here is focused on assessing the utility of this SCIL phase in the separation of peptides.

Experimental

Materials

All reagents used in the synthesis of the stationary phase (hydrogen hexachloroplatinic (IV) acid hydrate, 8-bromo-1-octene, trichlorosilane, chlorotrimethylsilane, trichlorooctylsilane, 1-butylimidazole, 2,6-lutidine, and anhydrous toluene) were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). The silica sorbent used is spherical 5 μm, 300 Å pore Symmetry® silica provided by the Waters Corporation (Milford, MA, USA).

The peptides used in the study (glycine–tyrosine (Gly-Tyr), valine–tyrosine–valine (Val-Tyr-Val), leucine enkephalin, methionine enkephalin, and angiotensin-II) were obtained from Sigma. Mobile phase components (HPLC-grade water and acetonitrile) were supplied by Sigma. TFA, sodium hydroxide, and nitromethane were obtained from Fisher Chemical Company (Fair Lawn, NJ, USA). Formic acid (FA) was obtained from Sigma.

Methods

Stationary phase synthesis

Stationary phase synthesis has been reported previously [45]. Briefly, the butyl imidazolium bromide-modified silica phase was prepared by hydrosilylation of the alkenylbromide followed by immobilization of the trichlorosilane ligand on to the surface of the silica substrate. The phase was then endcapped with chlorotrimethylsilane, and the butyl-imidazole was subsequently attached. An octylsilane-modified stationary phase [C8] was also prepared on the same lot of silica. In the case of the SCIL phase, elemental analysis (Galbraith Laboratories Inc., Knoxville, TN, USA) revealed that loading of the linker

was ~3.68 μmol/m^2, the loading of the endcapping agent was ~0.66 μmol/m^2, and the loading of the butylimidazolium cation was ~1.79 μmol/m^2. The loading for the octyl phase was found to be ~3.41 μmol/m^2. Both phases were packed into stainless steel HPLC columns (150 mm×4.6 mm ID; Waters Corporation).

HPLC analysis

All HPLC studies were carried at room temperature, using a Shimadzu (Columbia, MD, USA) LC-10AT solvent pump and a Shimadzu (Columbia, MD, USA) SPD-10A UV detector set at 214 nm. Chromatographic retention data were recorded with Shimadzu Class-VP software. Sample introduction was accomplished with a Shimadzu SIL-10AF automatic sample injector fitted with a 20-μL loop. All experiments were performed in triplicate with flow rates of 1 mL/min with mobile phases composed of mixtures of acetonitrile and water (*v*/*v*). TFA or FA is added to both the water and acetonitrile in equal concentrations. All the peptides were prepared in concentration range of 0.004–0.5 g L^{-1} in HPLC-grade water and were refrigerated when not in use. Because of the difficulty in identifying a solute, which does not interact with this multimodal stationary phase, the void volume of the column was determined by measuring the weight difference of the column when filled with either dichloromethane or hexane [45]. The void volume of the column was 1.82 mL.

Capillary electrophoresis analysis

All capillary electrophoresis studies were carried out using a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA, USA) controlled by 32 Karat Software (v. 8.0). Detection was carried out using UV/Vis at 214 nm. Fused-silica capillaries (75 μm i.d.) purchased from Polymicro Technologies Inc. (Phoenix, AZ, USA) were used. The capillary was conditioned prior to initial use using NaOH (1 M) for 10 min at 10 psi, and followed by rinsing with deionized (DI) water for 10 min at 10 psi. All experiments were performed in triplicate by using background electrolytes composed of 10% acetonitrile and water (*v*/*v*) with varying TFA percentages (0.001–0.06% *v*/*v*). Nitromethane (1% *v*/*v*) was used as the electroosmotic flow marker. All injections were performed hydrodynamically (0.5 psi for 3 s). The capillary was rinsed with DI water for 10 min at a pressure of 10 psi between each run.

Results and discussion

A schematic for the phase that was used for the study is shown in Fig. 1. Previous work done in our group has reported cation loading ranging from 0.7 to 1.0 μmol/m^2 (% linker$_{Modified}$=20% to 30%) on a 100 Å pore silica [45, 51]. Interestingly, cation loading on the 300 Å pore silica used in this work is 1.8 μmol/m^2 (% linker$_{Modified}$=49%), which is significantly higher than reported previously. This may be attributed to steric effects at the mouth of the smaller pores and is consistent with increased loading of bulky pendant groups on larger pore silica reported previously [52].

Successful separation of five peptides under a time scale of less than 5 min was obtained using 0.005% TFA with 10% ACN under isocratic conditions as can be seen in Fig. 2a. From the chromatogram, it can be seen that, surprisingly, three peptides out of the five eluted before the void volume (1.82 mL), while the other two eluted after the void volume. To elucidate the mechanism responsible, it is convenient to first examine the retention behavior of the solutes eluting after the void volume.

Post-void volume eluting peptides

Effect of organic modifier on enkephalin retention

The retention of enkephalins is plotted as elution volume vs %ACN in 0.001% TFA shown in Fig. 3. As can be seen in the figure, increasing %ACN corresponds to a decrease in the elution volume, which is consistent with general reversed-phased behavior of peptides on hydrophobic phases (e.g., C-4, C-8, and C-18) [53–55]. However, further increase in acetonitrile concentration causes further decreases in elution volumes; at acetonitrile concentrations above 40%, the enkephalins elute before the void volume even with higher concentrations of TFA (~0.1%). This behavior is in stark contrast to the behavior of peptides on conventional alkyl phases where a minimum retention is observed at some intermediate organic modifier concentration [2, 53, 56, 57]. This behavior is also in contrast when compared with the behavior of basic peptides on a weak anion exchange phases [58] where increasing acetonitrile concentrations also causes an increase in elution times.

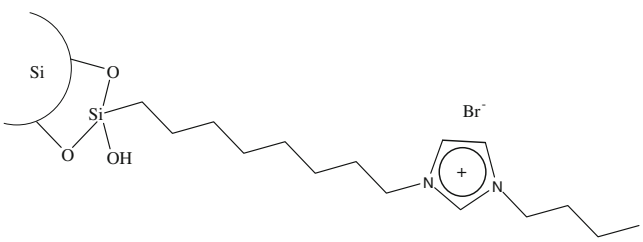

Fig. 1 SCIL stationary phase

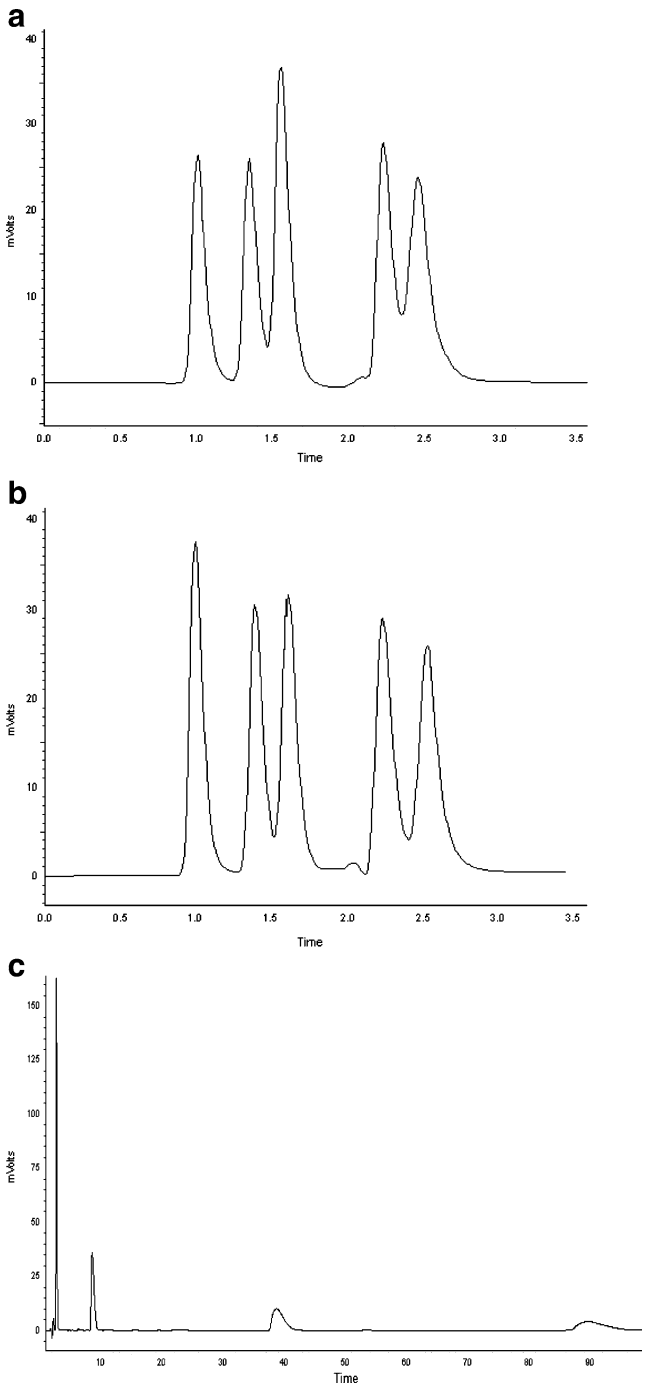

Fig. 2 Chromatogram obtained of peptide mixture using 10% ACN **a** with 0.005% TFA on SCIL phase (elution order is angiotensin-II, Val-Tyr-Val, Gly-Tyr, leucine enkephalin, and methionine enkephalin), **b** with 0.005% FA on SCIL phase (elution order same as in **a**), and **c** with 0.005%TFA on C_8 phase (elution order is Gly-Tyr, Val-Tyr-Val, methionine enkephalin, and leucine enkephaline). Angiotensin-II did not elute

Effect of TFA

In conventional reversed-phase chromatography of peptides using TFA as an ion-pairing agent, increased concentration

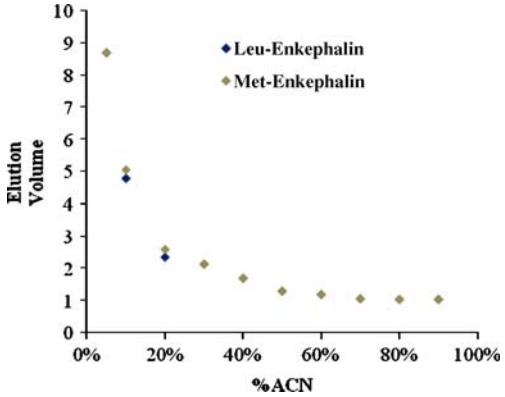

Fig. 3 A plot of the elution volume obtained on SCIL phase for the two enkephalins vs %ACN in 0.001%TFA

of TFA leads to increased retention [59]. A plot of log *k* for the enkephalins vs %TFA is shown in Fig. 4. As can be seen in the figure, increasing TFA concentration produces decreased retention for these two peptides. Interestingly, these results suggest that TFA is not acting as ion-pairing agent for these solutes on this SCIL phase under reversed-phase conditions. To further understand the role of TFA in separation of these peptides, the TFA was replaced with FA. Again, a successful separation of five peptides under a time scale of less than 5 min was observed in 0.005% FA with 10%ACN under isocratic conditions shown in Fig. 2b. As can be seen in the figure, virtually identical separation achieved using FA as a substitute for TFA is supporting evidence that TFA is not acting as ion-pairing agent in this separation. Further, the similarities of the chromatograms using FA and TFA suggest that the mobile phase anion does not play a significant role in the separation. To further understand the role of stationary phase in the separation of these peptides, the peptides were also separated on an alkyl phase (C-8 phase, 300 Å, 5 μm) prepared in-house using the best separation conditions (0.005% TFA, 10% ACN) found on the SCIL phase. Separation of peptides on the alkyl phase is shown in Fig. 2c. While none of the

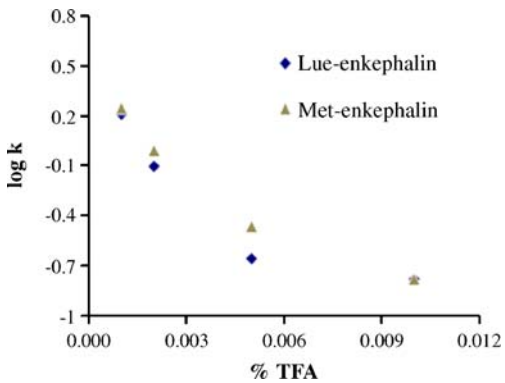

Fig. 4 A plot of the log *k* for the two enkephalins vs %TFA in 10% ACN

peptides eluted before the void volume on the C8 phase, they demonstrated a much wider range of elution volumes on the C8 phase than on the SCIL phase. Indeed, while angiotensin was the first to elute from the SCIL phase, it failed to elute after 200 min from the C8 phase under these mobile phase conditions. In addition, the elution order of the two enkephalins from the C8 phase was reversed relative to their elution order from the SCIL phase. Hence, the retention properties of the two phases are very different.

Pre-void volume eluting peptides

To elucidate the mechanism responsible for the solutes eluting before the void volume, it is helpful to examine the peptide structures. The first peptide eluting is angiotensin, which is the largest peptide followed by the tri- and dipeptide. While this elution order is consistent with size exclusion chromatography, these analytes are too small for a size exclusion mechanism. Further examination of the peptide structures reveals the presence of three amines in angiotensin-II whereas the di- and tripeptides contain only one. The concentration of TFA that is used for the study (0.001–0.1% *v*/*v*) is sufficient to protonate the three amine-containing angiotensin-II residues (Asp, Arg, and His).

The stationary phase used here has an imidazolium ring that is positively charged and could promote Donnan exclusion as in ion exclusion chromatography [60, 61] where similarly charged species to the phase are repelled, while oppositely charged and neutral species are retained. This kind of chromatography is predominately used in the separation of ions [60]. The higher charge on angiotensin-II (+3) charged relative to other peptides causes an increased exclusion of this peptide from the pores of the stationary phase. A plot of elution volume of angiotensin-II vs %TFA is shown in Fig. 5. As can be seen from the plot, increasing TFA concentration increases the elution volume, which may be attributed to masking of positive charges of the peptide and stationary phase by TFA anions. This behavior is consistent with an ion exclusion mechanism.

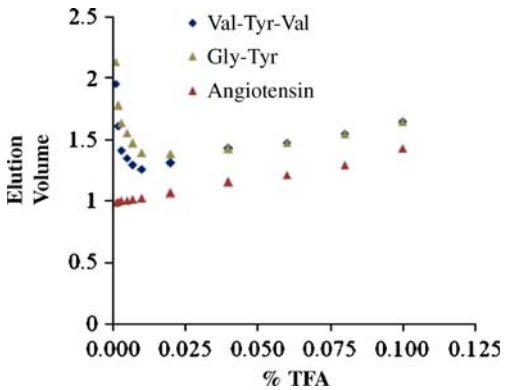

Fig. 5 A plot of the elution volume obtained on SCIL phase of angiotensin, Val-Tyr-Val, and Gly-Tyr vs %TFA in 10% ACN

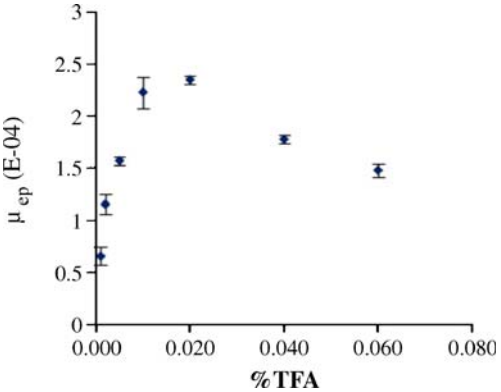

Fig. 6 A plot of the electrophoretic mobility of Val-Tyr-Val vs %TFA in 10% ACN

The next two eluting peptides are Val-Tyr-Val (tripeptide) and Gly-Tyr (dipeptide). Plots of the elution volumes of the di- and tripeptide vs %TFA are also shown in Fig. 5. As can be seen in Fig. 5, these two peptides' behavior with changing TFA concentrations is similar, further indicating the same mechanism that is involved for elution of both peptides before the void volume. At very low TFA concentrations, the elution volumes are high; at intermediate TFA concentrations, the elution volume decreases, but at higher concentrations, the elution volume increases again.

At low concentrations of TFA, the elution behavior of the di- and tripeptide may be attributed to neutralization of the carboxylate resulting from addition of the acidic TFA modifier. A plot of the electrophoretic mobility of the tripeptide in 10% ACN vs %TFA is shown in Fig. 6. In the absence of TFA, the peptide exists as the neutral zwitterion. Addition of small amounts of TFA begins to neutralize the carboxylate, and the peptide becomes more positively charged. As can be seen in the figure, the most dramatic change in electrophoretic mobility occurs in the same

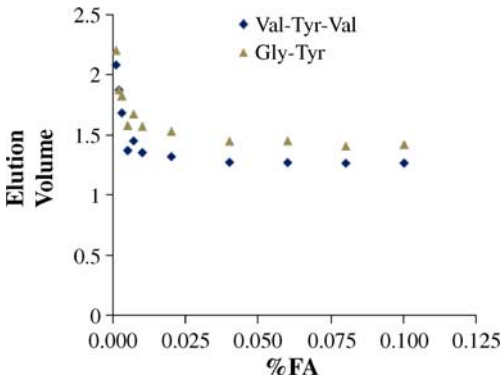

Fig. 7 A plot of the elution volume obtained on SCIL phase of Val-Tyr-Val and Gly-Tyr vs %FA in 10% ACN

region of TFA concentrations as in the chromatographic experiments in which the elution volume is decreasing (Fig. 5). As the TFA concentration is further increased in the electrophoresis experiment, the electrophoretic mobility starts to decrease, which may be attributed to peptide/TFA anion association. In the chromatographic case, the elution volume also begins to increase at higher concentrations of TFA; the increased elution volume of the two peptides may be attributed to the masking of the positive charges of the peptide and stationary phase by TFA anions.

Plots of elution volumes of tri- and dipeptide vs %FA is shown in Fig. 7. As can be seen in the figure, substitution of FA for TFA shows the same impact on elution volumes at low concentrations. However, in contrast to the TFA case, further increases in FA concentration do not lead to increased elution volume. This is likely because FA is a much weaker acid than TFA; hence, the low concentrations of its conjugate base are ineffective at counteracting the Donnan exclusion. Nevertheless, better separation of the two peptides is obtained in the presence of FA than in the presence of TFA across a range of acid concentrations.

Conclusions

The present study assesses the role of a SCIL stationary phase in the separation of peptides. A successful separation of peptides using this phase is seen under isocratic conditions. Despite the presence of covalently attached cations in the stationary phase, reversed-phase behavior is observed. TFA does not seem to be playing the role of ion-pairing agent on this phase for these solutes; hence, FA may be substituted for TFA in these systems. The results obtained on the SCIL phase demonstrate that while the SCIL phase exhibits some reversed-phase character, electrostatic interactions dominate at high organic and/or low pH modifier concentrations.

Acknowledgments The authors gratefully acknowledge support from the Waters Corporation and the National Institutes of Health (R01 GM 067991-02A2).

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