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Short communication

Separation of fluorinated amino acids and oligopeptides from their non-fluorinated counterparts using high-performance liquid chromatography

Nu Xiao [a], Y. Bruce Yu [a,b,*]

[a] Department of Pharmaceutical Sciences, University of Maryland, Baltimore, MD 21201, United States
[b] Fischell Department of Bioengineering, University of Maryland, College Park, MD 20742, United States

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ABSTRACT

Chromatographic conditions for the separation of fluorinated amino acids and oligopeptides from their non-fluorinated counterparts were explored. The separation of six pairs of analytes, including both aromatic and aliphatic fluorocarbons, was investigated at various temperatures using both hydrocarbon and fluorocarbon columns and eluents. Our results show that when hydrocarbon eluents are used, fluorocarbon column provides better separation of fluorinated amino acids or oligopeptides from their non-fluorinated counterparts; when fluorocarbon eluents are used, hydrocarbon column provides better separation of fluorinated amino acids or oligopeptides from their non-fluorinated counterparts. These chromatographic behaviors reflect the fluorophilicity possessed by fluorinated amino acids and oligopeptides.

1. Introduction

Although rarely existing in nature [1], organofluorine compounds are finding increasing applications in a wide range of biological and medical sciences, such as biochemistry [2], medicinal chemistry [3], pharmaceutical chemistry [4], green chemistry [5], biotechnology [6], drug delivery [7] and diagnostic imaging [8]. As is the case with any class of organic molecules, separation is an important consideration for organofluorine compounds [9]. Heavily fluorinated molecules have unique partition properties between fluorocarbon solvents and hydrocarbon solvents [10]. This feature has been exploited for the extraction and separation of compounds with multiple fluorine atoms, using either perfluorocarbon solvent extraction [11] or fluorous silica-gel chromatography [12].

The vast majority of fluorinated drugs contain just a few fluorine atoms [3,4]. In this work, we explore the separation of lightly fluorinated amino acids and oligopeptides from their non-fluorinated counterparts using high-performance liquid chromatography (HPLC). This problem arises during our work on developing fluorinated analogs of peptide drugs. We investigated several aspects of the separation of fluorinated amino acids and oligopeptides from their non-fluorinated counterparts, including: aromatic vs. aliphatic fluorocarbons, fluorocarbon (F-) vs. hydrocarbon (H-) columns, fluorocarbon (F-) vs. hydrocarbon (H-) eluents, and temperature. Both fluorocarbon columns and fluorocarbon eluents have been investigated for the separation of lightly fluorinated compounds [13–15]. However, to the best of knowledge, there has been no prior report in which fluorinated eluents, columns and analytes are compared side-by-side with their non-fluorinated counterparts.

We investigated six pairs of analytes, **2**/**1**, **4**/**3**, **6**/**5**, **8**/**7**, **10**/**9** and **12**/**11**, which can be divided into three groups: the aromatic fluorocarbon group ($C_6H_5 \rightarrow C_6H_4F$ substitution), which includes the **2**/**1** and the **4**/**3** pairs; the aliphatic fluorocarbon group ($CH_3 \rightarrow CF_3$ substitution), which includes the **6**/**5** and the **8**/**7** pairs; and the hydrocarbon control group ($H \rightarrow CH_3$ substitution), which includes the **10**/**9** and the **12**/**11** pairs. **8** stems from our effort on making fluorinated analogs of the peptide drug octreotide (Sandostatin®). Fig. 1 shows the structures of the 12 analytes.

For the separation of each pair of analytes, we used an F-column that contains the n-C_8F_{17} group and an H-column that contains the n-C_8H_{17} group. For each column, we used two fluorocarbon eluents, trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP),

Abbreviations: Cys, cysteine; DCM, dichloromethane; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; EtOH, ethanol; F%wt, fluorine weight percentage in an analyte or a solvent; Fmoc, fluorenylmethoxycarbonyl; HFIP, hexafluoroisopropanol; HOBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; ISP, isopropanol; Lys, lysine; MS, mass spectrometry; MW, molecular weight; Nle, norleucine; NMR, nuclear magnetic resonance; Nva, norvaline; Phe, phenylalanine; RPLC, reversed-phase liquid chromatography; tBu, *tert*-butyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; tfT, trifluorothreonine; Thr^{allo}, *allo*-L-threonine; t_R, retention time; Trp, tryptophan; Trt, trityl; Tyr, tyrosine; δ, chemical shift; Δt_R, retention time difference.

* Corresponding author at: Department of Pharmaceutical Sciences, University of Maryland, Baltimore, MD 21201, United States. Tel.: +1 410 705 7514.
E-mail address: byu@rx.umaryland.edu (Y.B. Yu).

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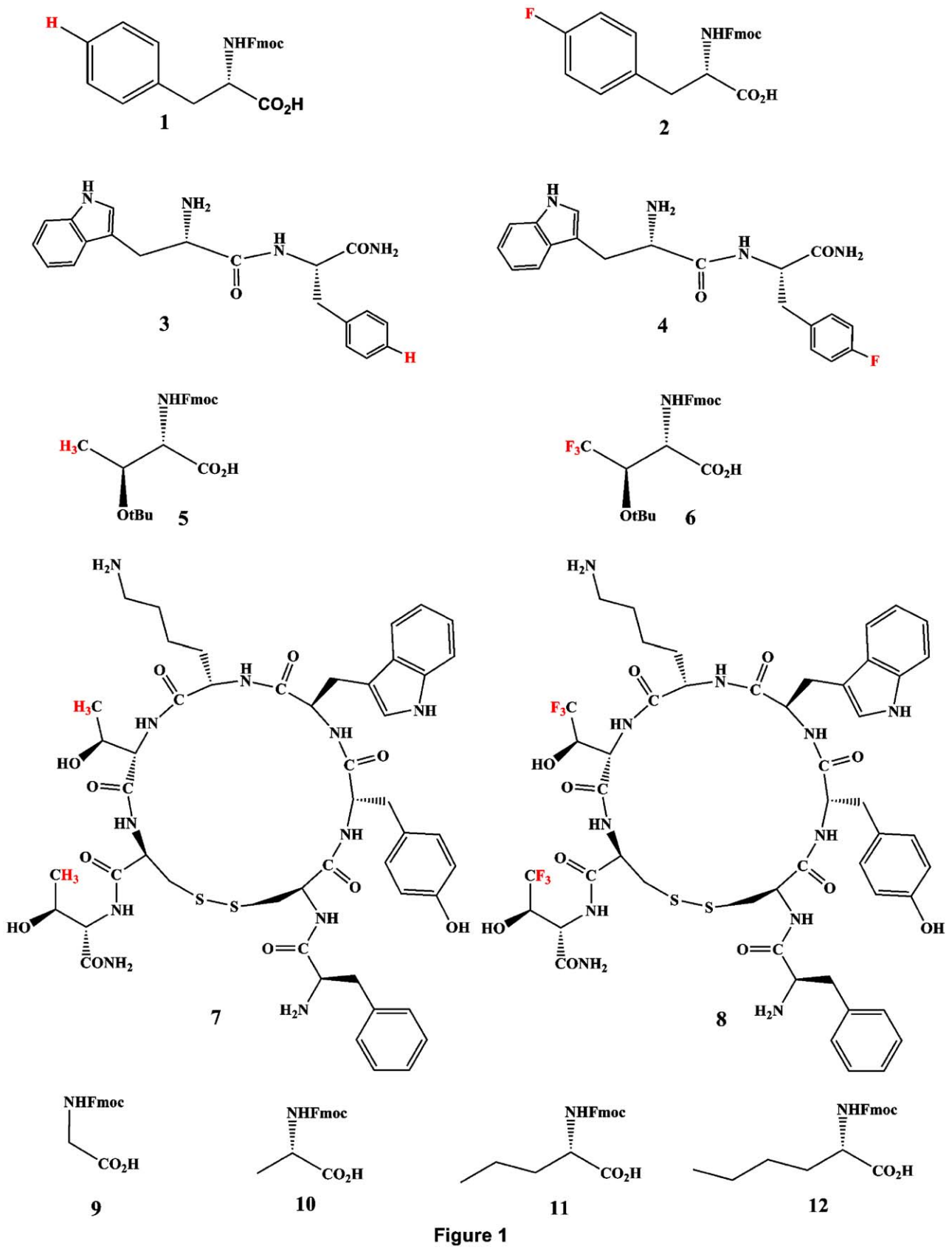

Fig. 1. Structures of analytes. The difference between a fluorinated analyte and its non-fluorinated analyte is highlighted in red. The 12 analytes can be divided into three groups: the $C_6H_5 \rightarrow C_6H_4F$ group (including the **2**/**1** pair and the **4**/**3** pair); the $CH_3 \rightarrow CF_3$ group (including the **6**/**5** pair and the **8**/**7** pair); and the $H \rightarrow CH_3$ group (including the **12**/**11** pair and the **10**/**9** pair). We used protected version of the amino acids because free amino acids were not sufficiently retentive on the HPLC columns we used. Peptide sequences are: Trp-Phe (**3**), Trp-Phe(4-F) (**4**), D-Phe-*c*[Cys-Tyr-D-Trp-Lys-Thr^{allo}-Cys]-Thr^{allo}-amide (**7**) and D-Phe-*c*[Cys-Tyr-D-Trp-Lys-tfT-Cys]-tfT-amide (**8**). **7** and **8** are cyclized through intramolecular disulfide bond, as in octreotide.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

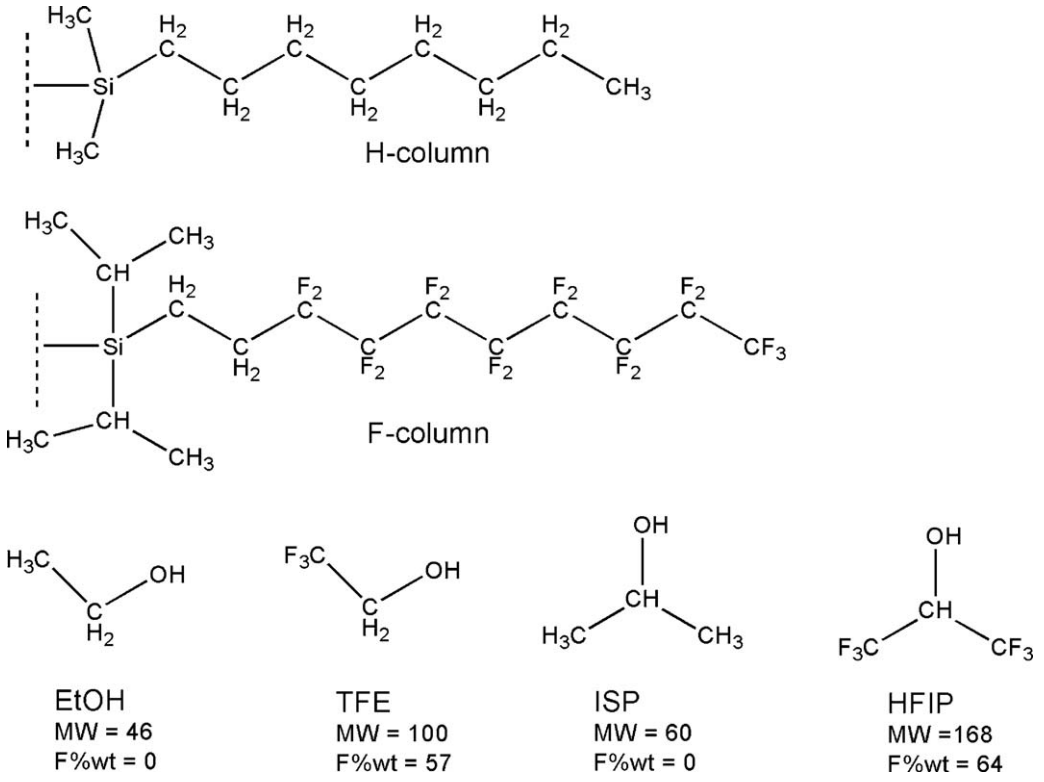

Fig. 2. Structures of stationary phase (columns) and mobile phase (eluents).

and their respective hydrocarbon counterparts, ethanol (EtOH) and isopropanol (ISP). For each eluent, chromatographic runs were conducted at temperatures ranging from 5 °C to 60 °C, at 5 °C increments. Fig. 2 shows structures of column stationary phases and structures of the eluents.

2. Results and discussion

2.1. Hydrophobicity analysis

Fig. 3 shows the retention behavior of analytes **1**–**12** in analytical RPLC. All four fluorinated compounds (**2**, **4**, **6** and **8**) were more retentive than their respective non-fluorinated counterparts (**1**, **3**, **5** and **7**). The retention time, t_R, provides a measure of the relative hydrophobicity of the analytes [16], as confirmed by the two control hydrocarbon pairs (**10**/**9** and **12**/**11**). According to this criterion, all four fluorinated analytes are more hydrophobic than their non-fluorinated counterparts. However, the difference in hydrophobicity caused by fluorination, gauged by retention time difference $\Delta t_R = t_R$(fluorinated) − t_R(non-fluorinated), varied considerably among the analytes even after normalization by the number of fluorine atoms or fluorine weight percentage (Table 1). This demonstrates that the extent of hydrophobicity elevation via fluorination depends on molecular context, which can outweigh the number of H → F substitutions. This is shown by the **6**/**5** and **8**/**7** pairs: even though **6** contains only one tfT residue (one $CH_3 \rightarrow CF_3$ substitution) while **8** contains two tfT residues (two $CH_3 \rightarrow CF_3$ substitutions), Δt_R is larger for the **6**/**5** pair than for the **8**/**7** pair, with or without fluorine content normalization (Table 1). Clearly, hydrophobicity elevation through fluorination is not determined solely by the number of H → F substitutions.

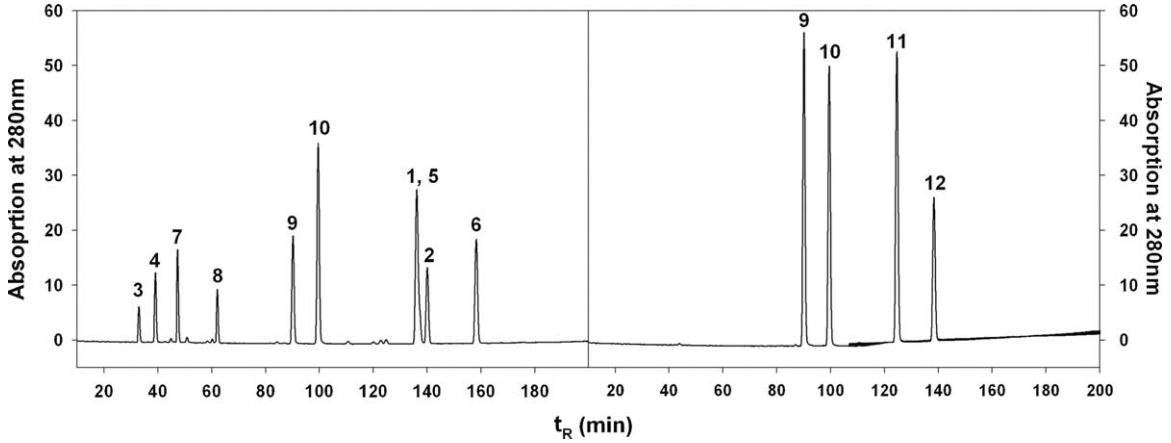

Fig. 3. Hydrophobicity analysis via RPLC. Analytes **1**–**10** were co-injected in one batch and analytes **9**–**12** were co-injected in another batch. Analytes **1** and **5** co-eluted under this condition.

Table 1
Characteristics of analytes.

Analytes	MW (Da)	n(F)[a]	F%wt[b]	Δt_R[c] (min)	Δt_R/n(F) (min)	Δt_R/F%wt (min)
1	387	0	0	3.91	3.91	0.83
2	405	1	4.7			
3	351	0	0	6.13	6.13	1.20
4	369	1	5.1			
5	397	0	0	22.11	7.37	1.75
6	451	3	12.6			
7	1048	0	0	14.70	2.45	1.48
8	1156	6	9.9			
9	297	0	0	9.35	N/A	N/A
10	311	0	0			
11	339	0	0	13.77	N/A	N/A
12	353	0	0			

[a] Number of fluorine atoms in an analyte.
[b] Fluorine weight percentage in an analyte (=19 × n(F)/MW).
[c] Retention time difference between an analyte pair in RPLC (Kromasil-C_{18} column).

2.2. Separation analysis

Separation profiles of the six analytes pairs, **2**/**1**, **4**/**3**, **6**/**5**, **8**/**7**, **10**/**9** and **12**/**11** are shown in Figs. 4–9, respectively. Our analyses of their separation profiles focus on the magnitude of Δt_R (degree of separation), the sign of Δt_R (order of elution), and the temperature dependency of Δt_R.

The effect of columns. For a given pair of analytes, the choice of column, H- or F-, has some effect on the magnitude of Δt_R, but no effect of the sign of Δt_R. The only exception is the **8**/**7** pair (Fig. 7):

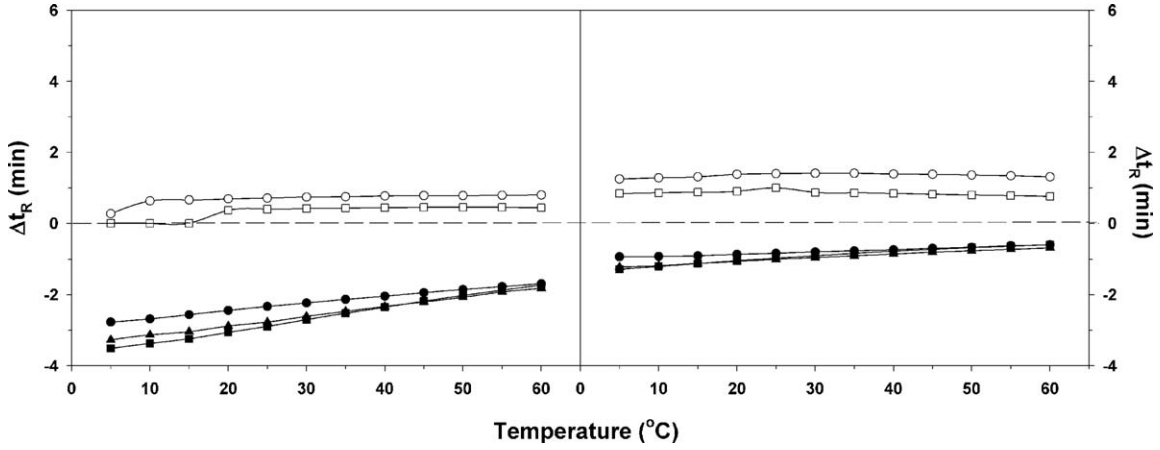

Fig. 4. Separation analysis of the **2**/**1** pair. $\Delta t_R = t_R(\mathbf{2}) - t_R(\mathbf{1})$. Left panel: H-column; right panel: F-column. (○) Water/EtOH eluent system; (●) water/TFE eluent system; (□) water/ISP eluent system; (■) water/HFIP eluent system; (▲) water/TFE:HFIP (1:1) eluent system.

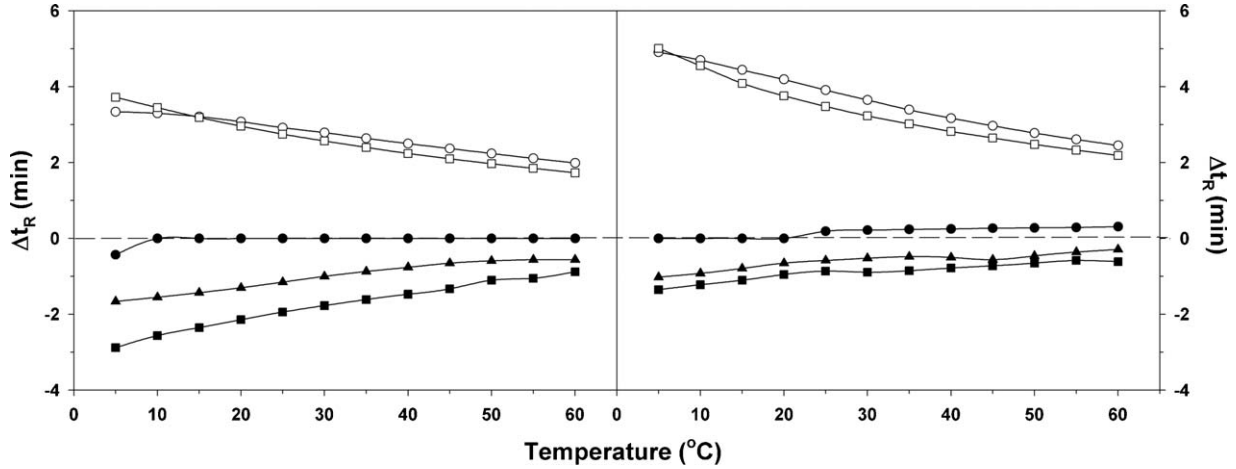

Fig. 5. Separation analysis of the **4**/**3** pair. $\Delta t_R = t_R(\mathbf{4}) - t_R(\mathbf{3})$. Left panel: H-column; right panel: F-column. (○) Water/EtOH eluent system; (●) water/TFE eluent system; (□) water/ISP eluent system; (■) water/HFIP eluent system; (▲) water/TFE:HFIP (1:1) eluent system.

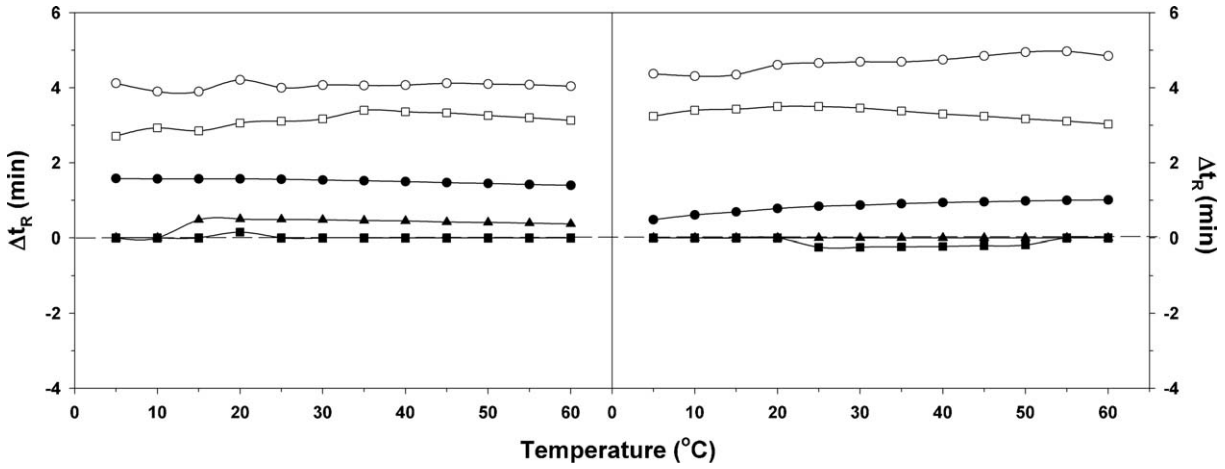

Fig. 6. Separation analysis of the **6**/**5** pair. $\Delta t_R = t_R(\mathbf{6}) - t_R(\mathbf{5})$. Left panel: H-column; right panel: F-column. (○) Water/EtOH eluent system; (●) water/TFE eluent system; (□) water/ISP eluent system; (■) water/HFIP eluent system; (▲) water/TFE:HFIP (1:1) eluent system.

when TFE was used as eluent on H-column, the sign of Δt_R was reversed as the temperature increased from 5 °C to 60 °C. Even in this case (**8**/**7**, TFE), the magnitude of Δt_R remained small after sign reversal.

The effect of eluents. For a given pair of analytes, the choice of eluents has significant, even dramatic, effects on Δt_R. The most dramatic effect is with the aromatic fluorocarbon group (**2**/**1** and **4**/**3**): when the eluent was switched from H- to F-solvents, the sign of Δt_R was reversed. As a general trend, Δt_R decreased steadily with the MW and F%wt of eluents for the two fluorocarbon groups (**2**/**1** and **4**/**3**; **6**/**5** and **8**/**7**), regardless of columns and temperature. In contrast, for the hydrocarbon group (**10**/**9** and **12**/**11**), Δt_R

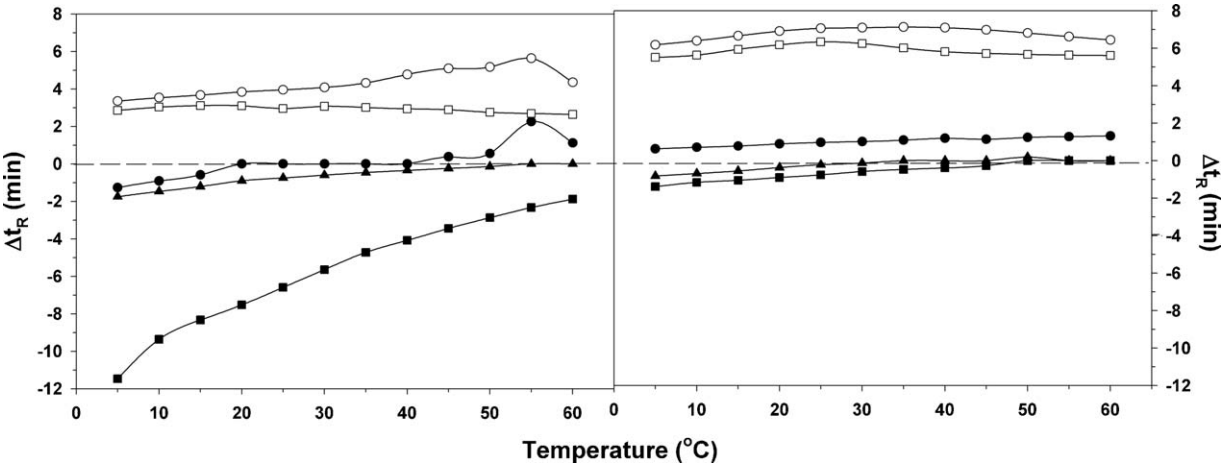

Fig. 7. Separation analysis of the **8**/**7** pair. $\Delta t_R = t_R(\mathbf{8}) - t_R(\mathbf{7})$. Left panel: H-column; right panel: F-column. (○) Water/EtOH eluent system; (●) water/TFE eluent system; (□) water/ISP eluent system; (■) water/HFIP eluent system; (▲) water/TFE:HFIP (1:1) eluent system.

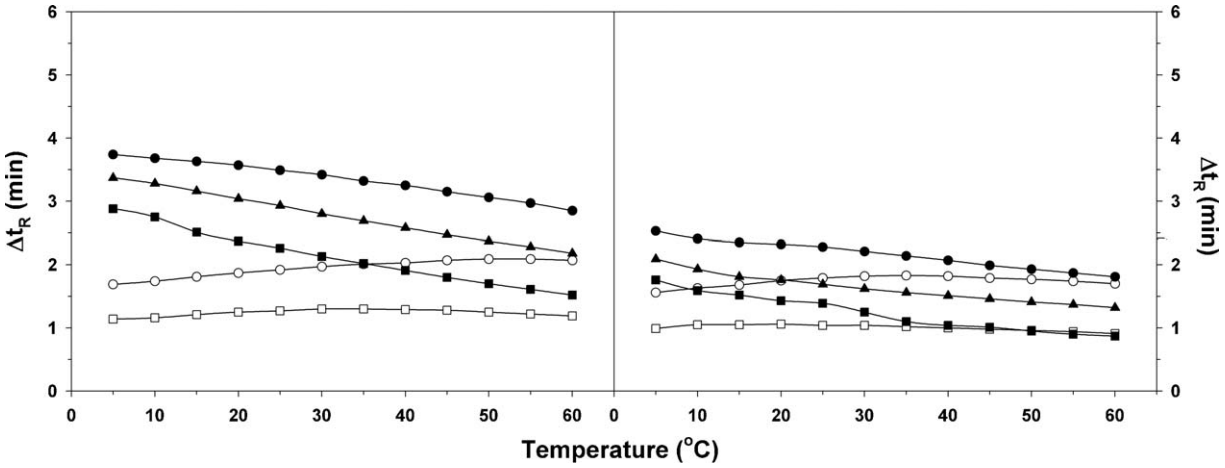

Fig. 8. Separation analysis of the **10**/**9** pair. $\Delta t_R = t_R(\mathbf{10}) - t_R(\mathbf{9})$. Left panel: H-column; right panel: F-column. (○) Water/EtOH eluent system; (●) water/TFE eluent system; (□) water/ISP eluent system; (■) water/HFIP eluent system; (▲) water/TFE:HFIP (1:1) eluent system.

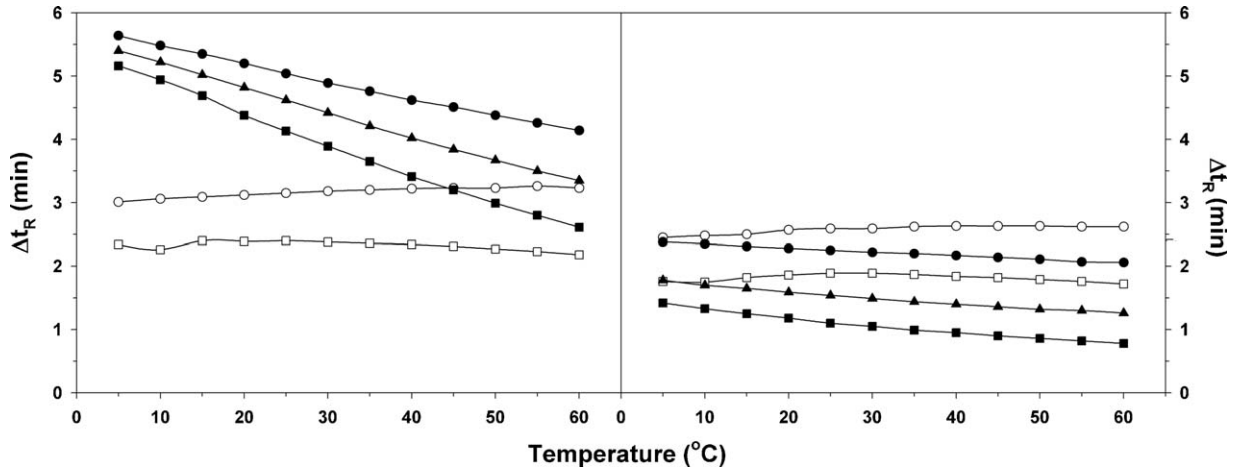

Fig. 9. Separation analysis of the **12**/**11** pair. $\Delta t_R = t_R(\mathbf{12}) - t_R(\mathbf{11})$. Left panel: H-column; right panel: F-column. (○) Water/EtOH eluent system; (●) water/TFE eluent system; (□) water/ISP eluent system; (■) water/HFIP eluent system; (▲) water/TFE:HFIP (1:1) eluent system.

increased when the eluent is switched from H- to F-eluents when H-column is used.

The effect of temperature. Temperature has various effects on the magnitude of Δt_R with no clear pattern. However, with the exception of the **8**/**7** pair on H-column with TFE as eluent, temperature has no impact on the sign of Δt_R for any analyte pair we investigated.

Fluorocarbons vs. hydrocarbons. Even though analytes **2**, **4**, **6**, **8**, **10** and **12**, are all more hydrophobic than their respective counterparts, **1**, **3**, **5**, **7**, **9** and **11**, there are significant differences between hydrocarbons and fluorocarbons. For the two hydrocarbon pairs (**10**/**9** and **12**/**11**), the more hydrophobic ones (**10** and **12**) were always eluted later than their less hydrophobic counterparts (**9** and **11**), i.e., $\Delta t_R > 0$ under all conditions. However, for the four fluorocarbon pairs (**2**/**1**, **4**/**3**, **6**/**5** and **8**/**7**), the more hydrophobic ones (**2**, **4**, **6** and **8**) sometimes were eluted earlier than their less hydrophobic counterparts (**1**, **3**, **5** and **7**), i.e., $\Delta t_R < 0$ under certain conditions. Such reversal of elution order happened to every fluorinated analyte pair on both H- and F-columns. The common feature is that in each case where the elution order was reversed, F-eluents were used.

Aromatic vs. aliphatic fluorocarbons. Among fluorocarbons, there are also differences between aromatic fluorocarbons and aliphatic fluorocarbons. Elution order reversal happens much more readily for aromatic fluorocarbons than for aliphatic fluorocarbons (Figs. 4–7). Further, after reversal, the magnitude of Δt_R was much larger for aromatic fluorocarbons than for aliphatic fluorocarbons. This is an interesting observation considering that F-eluents themselves are aliphatic fluorocarbons.

2.3. Fluorophilicity of lightly fluorinated compounds

It is well known that heavily fluorinated compounds have affinity toward each other, the so-called fluorophilicity [17]. Our work shows lightly fluorinated amino acids and oligopeptides also possess fluorophilicity. First, fluorinated analytes **2**, **4**, **6** and **8** demonstrate affinity toward F-column. When H-eluents were used, the magnitude of Δt_R increased as the column was switched from H- to F- (Figs. 4–7), indicating that a fluorinated analyte becomes more retentive on the F-column compared with its non-fluorinated counterpart. Second, fluorinated analytes **2**, **4**, **6** and **8** demonstrate affinity toward F-eluents. When the eluents were switched from H- to F-, Δt_R decreased steadily with the F%wt of the eluents (Figs. 4–7), regardless of column, indicating that a fluorinated analyte becomes less retentive on both H- and F-columns, compared with its non-fluorinated counterpart when F-eluents are used. Third, the two aromatic fluorocarbon analytes, **2** and **4**, demonstrate greater affinity toward F-eluents than toward F-column. When the eluents were switched from H- to F-, the sign of Δt_R (elution order) of the **2**/**1** and the **4**/**3** pairs was reversed in most cases, even when F-column was used, indicating that **2** and **4** prefer F-eluents over F-column.

The control hydrocarbon compounds demonstrate the expected aversion toward fluorocarbons (fluorophobicity). For these two analyte pairs, **10**/**9** and **12**/**11**, when H-eluents were used, Δt_R decreased as the column was switched from H- to F-, showing aversion toward F-column by the extra $-CH_2-$; when H-column was used, Δt_R increased as the eluents were switched from H- to F-, showing aversion toward F-eluents by the extra $-CH_2-$.

3. Conclusion

Chromatographic separation is a balance of complex inter- and intra-molecular interactions involving analytes, eluents, and columns. Generally speaking, optimal separation requires judicious choice of eluents, column and temperature. For a pair of lightly fluorinated amino acid or oligopeptide and its non-fluorinated counterpart, eluents have much more pronounced effect on their separation than columns and temperature. When hydrocarbon eluents are used, fluorocarbon column provides better separation of fluorinated amino acids or oligopeptides from their non-fluorinated counterparts; when fluorocarbon eluents are used, hydrocarbon column provides better separation of fluorinated amino acids or oligopeptides from their non-fluorinated counterparts. These chromatographic behaviors reflect the fluorophilicity possessed by fluorinated amino acids and oligopeptides.

For the two pairs of hydrocarbon analytes (**10**/**9** and **12**/**11**), the best separation condition is achieved with H-column and the water/trifluoroethanol eluent system. This improved separation in comparison with the water/ethanol eluent system is apparently caused by the aversion of hydrocarbons toward F-eluents.

4. Experimental

4.1. Chemicals, materials and instrumentation

Amino acids and resin. All amino acids have the *L*-configuration (i.e., 2*S*) unless otherwise specified. (2*S*, 3*S*)-*N*-Fmoc-*O*-*tert*-butyl-threonine (**5**) was purchased from BACHEM. (2*S*, 3*R*)-4,4,4-Trifluoro-*N*-Fmoc-*O*-*tert*-butyl-threonine (**6**) was synthesized

and purified by us as described in a previous publication [18]. Fmoc-norvaline (**11**) and Fmoc-norleucine (**12**) were purchased from Aapptec. Other Fmoc-protected amino acids and rink amide MBHA resin (0.65 mmol/g, 100–200 mesh) were purchased from Novabiochem. All purchased amino acids were used without further purification.

Eluents. EtOH was from Sigma–Aldrich (spectrophotometric grade); ISP was from EMD (HPLC grade); TFE and HFIP were from Oakwood Products (reagent grade). EtOH and ISP were used as purchased. TFE and HFIP were distilled before usage.

Columns. For separation studies, H-column, Zorbax Eclipse XDB-C_8 (4.6 mm × 150 mm, 5 μm); F-column, FluoroFlash® (4.6 mm × 150 mm, 5 μm). For hydrophobicity analysis, Kromasil-C_{18} (2.1 mm × 150 mm, 5 μm). For purity analysis, Zorbax XDB-C_{18} (4.6 mm × 250 mm, 5 μm). For chiral purity analysis, ChiraDex (4.6 mm × 250 mm, 5 μm). For peptide purification, Zorbax C_8 preparative column (21.2 mm × 250 mm, 5 μm).

Instrumentation. HP1200 liquid chromatography system (Agilent Technologies); JEOL ECX 9.4T NMR spectrometer (^{19}F 367 MHz); LCQ Man-O.2.2 mass spectrometer.

4.2. Peptide synthesis and purification

Peptides (**3**, **4**, **7** and **8**) were made using Fmoc solid-phase chemistry on rink amide MBHA resin. Except Cys and tfT, all coupling reactions were conducted using the Liberty microwave peptide synthesizer (5 eq. Fmoc-AA-OH, 4.5 eq. DIC, 5 eq. HOBt, 5 eq. DIPEA in DMF, 75 °C, 200 s). Cys and tfT are prone to racemization and their manual incorporation into peptides used the following conditions: Cys, 5 eq. Fmoc-Cys(Trt)-OH, 4.5 eq. DIC, 5 eq. HOBt, no base, r.t., 3 h; tfT, 3 eq. Fmoc-tfT(OtBu)-OH, 2.7 eq. DIC, 3.3 eq. HOBt, no base, 0.5 eq. $CuCl_2{\cdot}2H_2O$ in DMF/DCM (1/1), 0 °C, 16 h. $CuCl_2$ was added to reduce racemization [18]. Cyclic peptides (**7** and **8**) were made by crosslinking the two cysteine residues in each peptide (intramolecular S-S bond) in 10 mM ammonium acetate aqueous solution containing 17% DMSO (pH 7.0).

The peptides were purified using preparative reversed-phase liquid chromatography (RPLC). The purity of each peptide was verified using analytical reversed-phase and chiral liquid chromatography. The molecular weight (MW) of each peptide was verified using mass spectrometry (MS). The fluorinated octapeptide (**8**) was further characterized by ^{19}F NMR spectroscopy. For HPLC, MS and NMR data on **3**, **4**, **7** and **8**, see Supporting Information.

4.3. HPLC

For hydrophobicity analysis, we followed the method developed by Hodges and co-workers for measuring the relative hydrophobicity of peptides and amino acids [14]. The chromatographic conditions were: eluent A: 0.2% TFA in water; eluent B: 0.2% TFA in CH_3CN; gradient: 0.25% B/min, starting from 10% B; flow rate: 0.3 mL/min; column temperature: 25 °C. Room temperature was set at 20 °C. To avoid overcrowding the chromatogram, injections were conducted in two batches: analytes **1**–**10** were co-injected and analytes **9**–**12** were co-injected.

For separation analysis, the chromatographic conditions were: eluent A: 0.1% TFA in water; eluent B: 0.1% TFA in EtOH, or TFE, or ISP, or HFIP, or TFE/HFIP mixture (1:1); gradient: 1% B/min; flow rate: 0.5 mL/min; column temperature: 5–60 °C. For column temperatures below 20 °C, room temperature was set at 5 °C. For column temperatures between 20 °C and 60 °C, room temperature was set at 20 °C. Each pair of analytes was co-injected. For detailed gradient conditions of each pair, see Table S1 of Supporting Information.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jfluchem.2009.11.025.

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