ELSEVIER

Journal of Chromatography A, 923 (2001) 107–117

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

# Reversed-phase separation of basic tricyclic antidepressants using buffered and fluoroform-enhanced fluidity liquid mobile phases

Jun Zhao, Susan V. Olesik*

*Ohio State University, Department of Chemistry, 100 W. 18th Ave., Columbus, OH 43210-1153, USA*

Received 7 July 2000; received in revised form 18 April 2001; accepted 25 April 2001

**Abstract**

In an effort to expand the range of applications of enhanced-fluidity liquid chromatography (EFLC) to strongly polar and basic analytes, fluoroform ($CHF_3$) was investigated as a fluidity-enhancing agent. Fluoroform was chosen due to its high polarity, low viscosity and chemical inertness toward water and basic analytes. A group of representative basic compounds, tricyclic antidepressants, covering a wide range of polarity was chosen as model compounds. Their retention behavior on a $C_{18}$ stationary phase in methanol/phosphate buffer and methanol/phosphate buffer/$CHF_3$ mobile phases was characterized. The chromatographic performance with mobile phase conditions of different pH, with and without $CHF_3$ addition and with addition of triethylamine was studied. The advantages of using $CHF_3$ enhanced and buffered mobile phases were shown in the much improved chromatographic performance, such as shortened analysis time, increased efficiency, lower pressure drop and improved selectivity. Furthermore, this study demonstrated for the first time, that a commercial instrument could be readily utilized for EFLC separations which greatly expands the application range of the EFLC technique and chromatographic instrumentation. 

*Keywords:* Enhanced-fluidity liquid; Mobile phase composition; Fluoroform; Tricyclic antidepressants

## 1. Introduction

The separation of strongly polar and basic compounds has remained one of the most difficult tasks in reversed-phase high-performance liquid chromatography (HPLC). Due to their high column efficiency, ease of use, flexibility and wide applicability, silica bonded phases are by far the most popular choices for such tasks. However, there are still many problems associated with surface residual silanol groups, such as peak broadening, poor peak shape and irreproducible retention.

Enhanced-fluidity liquid chromatography (EFLC) has been continuously growing because it is capable of solving a wide range of separation problems. Liquid mixtures with enhanced fluidity that have been characterized most comprehensively to date have all employed carbon dioxide ($CO_2$) as the fluidity modifier, including mixtures such as methanol/$CO_2$, methanol/$H_2O$/$CO_2$ and THF/$CO_2$ [1–9]. Enhanced-fluidity liquid mixtures maintain solvent strengths similar to that of the pure organic component in the mixture even when as much as 40–50 mole% fluidity modifier is added and the

*Corresponding author. Tel.: +1-614-292-0733; fax: +1-614-292-1685.

*E-mail address:* olesik.1@osu.edu (S.V. Olesik).

0021-9673/01/$ – see front matter 
PII: S0021-9673(01)00922-0

viscosity is substantially reduced with the addition of a liquified gas. Carbon dioxide has several indisputable advantages as the fluidity modifier, such as low viscosity, low critical parameters and reasonably low cost. However $CO_2$ has inherent limitations which include formation of carbonic acid when combined with water, reaction with basic compounds such as primary and secondary amines [10,11] and limited solvating power. These limitations have prevented $CO_2$-based EFLC from a wider application range, especially for strongly basic analytes. Two consequences of $CO_2$ addition to methanol/$H_2O$ mixtures that affect the mobile phase pH and therefore the ionization of analytes include: the formation of carbonic acid and the reduction of the dielectric constant of the mixture [12].

Fluoroform ($CHF_3$) was chosen as the target fluidity-enhancing agent. It not only has the similar advantages as $CO_2$, such as even lower critical parameters, ($T_c$=299.2 K and $p_c$=47.94 atm [13]) and lower viscosity, but also possesses attributes that $CO_2$ lacks, such as no reaction with water or basic compounds. $CHF_3$ is polar and polarizable, with a dielectric constant within the range of 6–7 at the temperature of 303 K and the pressure of more than 100 atm [14]. It is chemically inert, non-flammable and has low toxicity. Recent phase diagram studies revealed that $CHF_3$ is markedly more soluble in methanol/$H_2O$ mixtures than $CO_2$ [15]. In addition, it is an environmentally-friendly Freon® [16].

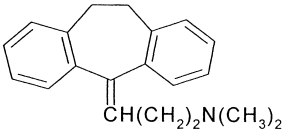
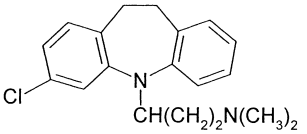
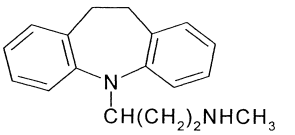
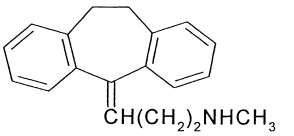
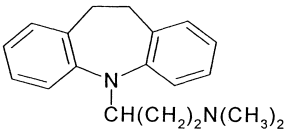
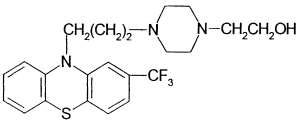
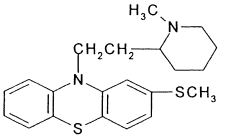
The use of $CHF_3$ in EFLC was initially illustrated in a reversed-phase separation of triazine herbicides [17]. $CHF_3$-enhanced liquid mobile phases achieved better chromatographic performance because it exhibited even lower viscosity than that of liquid mobile phases with a comparable amount of $CO_2$ addition. A combination of employing a phosphate buffer and $CHF_3$ in the mobile phase achieved the best results for triazine herbicides using reversed-phase HPLC. It was suggested that $CHF_3$ may be preferred for the separation of moderately to highly basic analytes. However, most triazine compounds are moderately polar and very weak bases with p$K_a$ values of 2 to 4 [17]. The separation of strongly polar and basic compounds using EFLC has not been demonstrated previously.

In this study, reversed-phase separations of seven basic tricyclic antidepressants (TCAs) using buffered mobile phases with and without $CHF_3$ addition were compared. Due to the strong polarity and basicity of these analytes, buffered mobile phases must be used to control their ionization throughout the experiments.

Table 1 shows the structures, names, designated acronyms, p$K_a$ and log $P_{ow}$($P_{ow}$ is octanol/water partition coefficient) values of the seven tricyclic antidepressants (TCAs) that were used in this study [18]. The first five compounds are commonly-used tricyclic antidepressants, covering a range of polarity while having very similar structures. The last two compounds are phenothiazine derivatives that are usually given in conjunction with TCAs to treat depressed patients [19,20]. Many specific HPLC methods have been developed for the analysis of TCAs in different biological systems. However, simultaneous separation of tricyclic antidepressants is difficult, not only due to their strongly polar and basic nature, but also due to their structural similarity and yet a wide range of polarity. In fact, as a group of representative basic compounds, TCAs are often used to evaluate the performance of stationary phases specifically designed for basic compounds. Bogusz et al. compared the chromatographic behavior of five TCAs using base-deactivated columns from different column manufacturers [21]. The study showed that the retention of these TCAs varied significantly from column to column even with the ''same'' stationary phase.

To date, all enhanced-fluidity liquid chromatography was performed using premixed mobile phases. Premixed enhanced-fluidity liquid mobile phases have several distinct advantages. Mobile phase composition is uniform throughout the experiment since there is no solvent evaporation or other contamination. Mobile phase compositions can be repeatedly made in a very accurate and precise manner. However, there are also several disadvantages. Because each syringe pump has a limited volume, such as 266 ml for a 260D ISCO pump (ISCO, Lincoln, NE, USA), one chromatographic run cannot exceed this maximum volume. In order to further expand the applicability of EFLC, a commercial instrument that functions as an SFC or an HPLC was utilized to perform EFLC.

Table 1
Structure and physical parameters of seven tricyclic antidepressants (TCA)[a]

| TCA | p$K_a$ | Log $P_{ow}$ | Structure |
|---|---|---|---|
| Amitriptyline hydrochloride (AM) | 9.4 (25°C) | 3.0 (pH 7.4) |  |
| Clomipramine hydrochloride (CL) | 9.3 (20°C) | 3.4 (pH 7.4) |  |
| Desipramine hydrochloride (DE) | 10.2 (24°C) | 1.4 (pH 7.4) |  |
| Nortriptyline hydrochloride (NO) | 9.7 | 1.7 (pH 7.4) |  |
| Imipramine hydrochloride (IM) | 9.5 (24°C) | 2.5 (pH 7.4) |  |
| Fluphenazine dihydrochloride (FL) | 3.9, 8.1 | 3.5 (pH 7.0) |  |
| Thioridazine hydrochloride (TH) | 9.5 (24°C) | [b] |  |

[a] Data from Ref. [19].
[b] Data were not available.

## 2. Experimental section

### 2.1. Materials

Seven tricyclic antidepressants, including amitriptyline hydrochloride (≥98%), clomipramine hydrochloride (≥98%), desipramine hydrochloride (≥98%), fluphenazine dihydrochloride (≥99.5%), imipramine hydrochloride (≥98%), nortriptyline hydrochloride (≥98%), thioridazine hydrochloride (≥99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Uracil (≥98%) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). HPLC-grade methanol was obtained from J.T.

Baker (Phillipsburg, NJ, USA) and specified at 100.0% purity with a $H_2O$ content of less than 0.01%. Triethylamine (≥99.5%) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Distilled $H_2O$ was deionized by a NANOpure II system (SYBRON/Barnstead Boston, MA, USA) with a resistivity of 17.8 to 18.3 MΩ. Electronic-grade Halocarbon 23 (99.95% purity fluoroform) without a helium pad was obtained from Air Products and Chemicals (Allentown, PA, USA) and was used as received. Impurities in fluoroform were specified as 500 volume ppm of air, 0.030 volume ppm of acidity as HCl and 10 weight ppm of $H_2O$.

Potassium dihydrogen phosphate (ACS certified, 99.7%) and dipotassium hydrogen phosphate (ACS certified, 99.2%) from Fisher Scientific (Fairlawn, NJ, USA), potassium hydroxide (pellets, ACS, 86.6%) from Jenneile Enterprises (Cincinnati, OH, USA) and phosphoric acid (85%) from Mallinckrodt Chemical (St. Louis, MO, USA) were used as received.

### 2.2. Instrumentation

The chromatographic system employed in this study was a Gilson SF3 Supercritical Fluid Chromatograph (Gilson Inc., Middletown, WI, USA). The system was configured for analytical-scale chromatography. Liquefied $CHF_3$ was pumped with Pump A (model 308 with a 10 SC pump head) with thermostated head. Premixed methanol/buffer mixtures were pumped with Pump B (model 306, with a 5 SC pump head), while pure methanol was pumped with Pump C (model 306 with a 5 SC pump head). Binary or tertiary mixing took place in a dynamic mixer (model 811C) with a 1.5 ml mixing chamber. Fixed loop injections (2 μl) were accomplished using a Rheodyne external loop injector (model 7725i) (Rheodyne L.P., Rohnert Park, CA, USA). Column temperature control was accomplished using a Gilson model 831 temperature regulator, with a maximum deviation less than ±0.5°C. An ice and water mixture was continuously circulated through the cooling coil of the Pump A with a Techne Tempunit® Thermoregulator (model TU-16D) (Techne Inc., Princeton, NJ, USA). Detection was accomplished at 220 nm using a model 151 variable wavelength UV detector with a high pressure flow cell (6 μl cell volume). Column outlet pressure was maintained using a model 821 pressure regulator. Instrument operation and data acquisition was achieved by Unipoint™ System control software version 1.80 (Gilson Inc. Middletown, WI, USA).

The analytical column was a Luna $C_{18}$ column (4.6 I.D.×150 mm long, packed with 5 μm particles) manufactured by Phenomenex (Torrance, CA, USA). The average pore diameter of the particles was 95±15 Å and the particle surface area was 440±30 $m^2/g$ with a carbon loading of 19.00±0.7%. The surface coverage of $C_{18}$ was 3.25±0.50 μmoles/$m^2$ and metal content was less than 55 ppm. The stated pH stability range for the column was specified as 1.5 to 10.0. A SecurityGuard™ cartridge (4×3.0 mm) with the same packing material (Phenomenex, Torrance, CA, USA) was directly connected to the inlet of the analytical column. A silica precolumn (BETASIL™ silica, 200 Å, 20×4.0 mm) from Keystone Scientific (Bellefonte, PA, USA) was placed before the injector to prevent dissolution of silica.

Individual stock solutions (1 mg/ml) were made by dissolving solid analytes in 80/20 (v/v) methanol/$H_2O$ in amber vials. Sample mixtures for chromatographic studies were prepared by mixing appropriate amount of stock solutions and diluting the mixture with 80/20 (v/v) methanol/$H_2O$ in an amber vial. The final concentrations of each analyte were from 0.03 to 0.05 mg/ml. The mixture was filtered through a 0.2 μm syringe filter (Whatman Inc., Clifton, NJ, USA). All solutions were stored in dark at 4°C when not in use.

Phosphate buffers of different pH were prepared by dissolving the appropriate quantity of $K_2HPO_4$ in pure water to make a stock solution of 20.0 m*M* (also including an appropriate amount of triethylamine if necessary), then adjusting the pH with either concentrated phosphoric acid or 20.0 m*M* KOH solution, in order to maintain the final concentration of $K^+$ as a constant of 20.0 m*M*. Buffer pH was measured before addition of the organic modifier. An Accumet 10 pH/mV meter from Fisher Scientific (Pittsburgh, PA, USA) was used for pH measurement. The pH meter was first calibrated at pH 7.00 using a standard buffer of potassium phosphate monobasic, potassium phosphate dibasic (Mallinckrodt-Baker Inc., Paris, KY, USA) and then at either pH 4.00 standard buffer

of potassium acid phthalate (Baxter Diagnostics Inc., Deerfield, IL, USA) or pH 10.00 standard buffer of boric aid, potassium borate (Mallinckrodt-Baker Inc., Paris, KY, USA) depending on the desired buffer pH. All measurements were made within ±0.01 pH unit.

### 2.3. Experimental procedure

The volumetric flow-rate was maintained constant at 0.50 ml/min. Column outlet pressure was held at 102 atm (1500 p.s.i.) by the regulation valve. All mixtures containing $CHF_3$ were well within the one phase region under experimental conditions [15]. The column inlet pressure, $p_1$, and outlet pressure, $p_2$, were continuously monitored and recorded. Injection of uracil (0.05 mg/ml) dissolved in methanol/$H_2O$ (80/20, v/v) was used to estimate the column void volume under different mobile phase conditions. All chromatographic runs were performed at 28.0±0.1°C. Reported data were averages of at least duplicates or triplicates. At least 15 column volumes of mobile phase were used to equilibrate the column after each mobile phase change. An additional 15 to 20 column volumes of mobile phase were used to equilibrate the chromatographic system when switching between LC and EFLC modes.

### 2.4. Data analysis

The chromatographic data were collected by Unipoint™ System control software, running on a Gateway™ model E-3200 Pentium™ II based personal computer. Different sampling frequencies were used depending on the retention times of the analytes. Data were analyzed by PeakFit™ version 4.06 (PeakFit Analysis Software, Jandel Scientific, San Rafael, CA, USA). The chromatographic parameters were determined by fitting the experimental data to an exponentially modified Gaussian distribution.

## 3. Results and discussion

### 3.1. Effect of mobile phase pH on the separation under HPLC and EFLC conditions

In reversed-phase HPLC, the retention of ionizable analytes is greatly influenced by their ionization states, which are governed by the pH of mobile phase. Therefore, it has become a common practice to employ an aqueous buffer in the mobile phase to control its pH and thus the ionization of the analytes.

In order to better examine the applicability of $CHF_3$-enhanced liquid mobile phases for basic compounds, a study of retention variation as a function of mobile phase pH under both LC and EFLC conditions was conducted. In liquid chromatography, mobile phase pH normally refers to the ''apparent'' pH, which is the pH of aqueous buffer before the addition of the organic modifier. However, since these mobile phases contain significant amounts of methanol, the mobile phase pH is expected to differ noticeably from the value of pure buffers. By utilizing the equations generated by Bosch et al. [22], which allow the calculation of p$K_a$ values in methanol/$H_2O$ mixtures for commonly-used buffers, the pH value of a phosphate buffer in a given methanol/$H_2O$ mixture was estimated using the Henderson–Hasselbalch equation. Calculations showed that for phosphoric acid in 80/20 (v/v) (0.64/0.36 mole fraction) methanol/water mixtures, it has a p$K_{a1}$ of 4.3 and p$K_{a2}$ of 9.6, which is in agreement with literature values [23,24], compared to 2.1 and 7.2 in pure water, respectively. Therefore, for the methanol/phosphate buffer of this composition, the actual pH is approximately 2 units above the pH in pure $H_2O$. The increased p$K_a$ values are attributed to the decreased dielectric constant [12]. Furthermore, the decrease in dielectric constant resulting from the addition of $CHF_3$ to this methanol/phosphate buffer mixture should also cause an increase in the p$K_a$ of buffer species and thus the mobile phase pH. This effect cannot be quantified without an estimate of the dielectric constant of methanol/buffer/$CHF_3$ mixtures. Since we were most interested in the effect on ionization of basic compounds, the ''apparent'' pH of the aqueous buffer was used throughout the remainder of this study.

Fig. 1A and B show plots of the retention factor, *k*, as a function of mobile phase pH using the following mobile phase conditions, respectively: 80.0/20.0 (v/v) methanol/aqueous buffer, i.e. 64.0/36.0 mole% methanol/20 m*M* phosphate buffer; 80/20 (v/v) methanol/20 m*M* phosphate buffer with

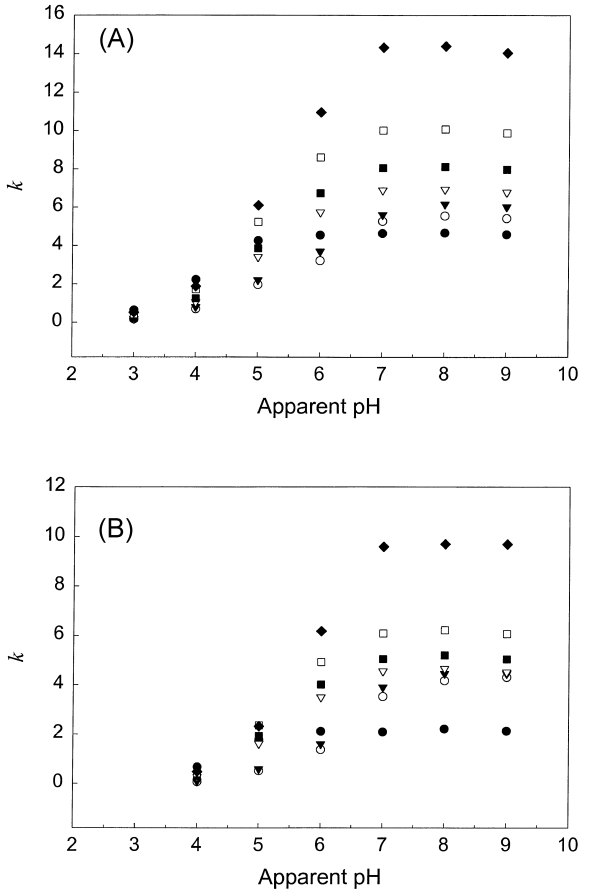



Fig. 1. Plots of retention factor $k$ as a function of mobile phase pH for seven TCAs under (A) LC condition: mobile phase of 64.0/36.0 mole% methanol/20 m*M* phosphate buffer; (B) EFLC condition: mobile phase of 53.4/30.0/16.6 mole% methanol/20 m*M* phosphate buffer/$CHF_3$. FL (●), DE (○), NO (▼), IM (▽), AM (■), CL (□), TH (♦).

16.6 mole% $CHF_3$ addition, i.e. 53.4/30.0/16.6 mole% methanol/20 m*M* phosphate buffer/$CHF_3$. Eq. (1) describes the variation of the retention factor of a weakly monoprotic base with mobile phase pH when it interacts with a typical reversed-phase surface, such as $C_{18}$ and $C_8$ stationary phase [25]:

$$k = \frac{k_B K_a [H^+] k_{BH^+}}{K_a + [H^+]} \quad (1)$$

$k$ is the observed retention factor of the analyte; $k_B$ and $k_{BH^+}$ are the retention factors of the neutral and fully ionized base, respectively; $K_a$ is the acid–base dissociation constant for weak acid $BH^+$ in the mobile phase. Clearly, the retention and selectivity of reversed-phase HPLC are strongly affected by the pH of the mobile phase for ionizable compounds. Ionization is also occurring in the enhanced-fluidity liquid mixtures and the addition of buffers affected the extent of ionization. A dramatic change in solute retention factor occurred at pH values close to the analyte's p$K_a$ value. At the high pH region, typically where pH was 2 units above its p$K_a$, the retention factor reached a maximum and remained nearly constant; at the low pH region, where pH is 2 units below its p$K_a$, minimum solute retention was observed in both cases since analytes were completely ionized.

The p$K_a$ values for these basic compounds were estimated from Fig. 1A and B to be approximately an apparent pH of 5, although their p$K_a$, values in pure water are 9–10. When alcohols, such as methanol and ethanol, are added to water the extent of ionization is normally suppressed for both acids and bases. As a general rule, p$K_a$ values in 60/40 (v/v) methanol/$H_2O$ are usually about one unit lower than in pure water [26]. For example, Vervoort et al. [27] determined p$K_a$ values of some basic drugs in 60/40 v/v% methanol at 37°C. The p$K_a$ for IM was determined to be 8.0 in 60/40 v/v% methanol at 37°C, as compared to 9.5 in pure water at 25°C. Because 80 vol% of methanol was present in this study, the p$K_a$ was further reduced. The decrease in p$K_a$ values was attributed to the presence of organic component, methanol, which lowered the dielectric constant of the mixed solvent and therefore suppress the solute ionization.

### *3.2. Comparison of HPLC separations at acidic pH (pH 3) and intermediate pH (pH 7)*

For the separation of basic compounds, it has been suggested that mobile phases of pH 3 should be considered first [28]. At this acidic pH, the basic compounds are completely ionized while any silanol groups on the support or stationary phase are protonated. Therefore, better peak shapes and narrower bands can often be achieved. However, since basic compounds at low pH are very polar, their retention

will often become very short and the chromatogram will lack adequate resolution, as illustrated in Fig. 1A. Higher proportions of $H_2O$ are needed in the mobile phase to increase retention and thus resolution. For the TCAs, baseline separation could not be achieved even when as much as 50 v/v% of phosphate buffer was used, while the retention time became extremely long, as shown in Fig. 2.

It is clear that with this methanol/buffer composition, separation of these basic drugs is amenable only in intermediate to high pH region (e.g. pH 7 and above), as shown in Fig. 1A and B. In this region, the ionization of these analytes is greatly suppressed, therefore showing much increased retention and better selectivity. Moreover, separation of basic drugs at pH 7 is preferred due to the high resolution achieved compared to that obtained with mobile phases at pH 8 and 9. The use of mobile phases with pH 8 or 9 did not increase retention, because it had minimal impact on ionization of these analytes, as shown in Fig. 1A and B. Since p$K_a$ values for these basic drugs were estimated to be around 5, under LC and EFLC mobile phase conditions most of these analytes should remain neutral at this pH, while the support silanol groups are mostly ionized. Therefore, silanol interactions with basic functionalities were also expected to be minimal.

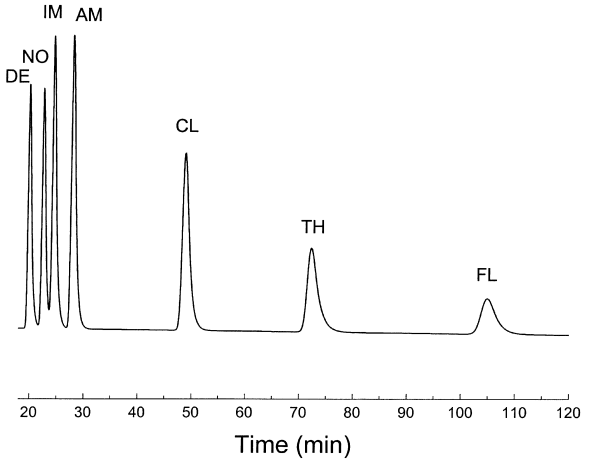



Fig. 2. Chromatogram of LC separation of seven TCAs using mobile phase of 30.9/69.1 mole% methanol/20 m*M* phosphate buffer (pH=3).

### *3.3. Comparison of LC and EFLC separations at pH 7*

For neutral compounds, Eq. (2) is often used to describe the variation of the retention factor with volume fraction of the organic component of the mobile phase [25,29,30]:

$$\log k = \log k_w + S\phi \quad (2)$$

$k_w$ is solute retention factor with pure water as the mobile phase; $\phi$ is the volume fraction of organic component; $S$ is a solute-dependent constant related to the solvent strength of the organic solvent. Experimentally determined $S$ and $\log k_w$ values can provide some insight into the selection of mobile phase compositions.

Since the ionization of the TCA compounds is greatly suppressed at pH 7 and above, Eq. (2) should describe the variation of retention for these basic analytes under both LC and EFLC conditions. Because $CHF_3$ is a compressible fluid, it is more reasonable to express mobile phase mixtures in terms of mole fraction, $\chi$ of each component [31]. The calculated slope in these studies will be designation $S'$ to denote the use of mole fraction rather than volume fraction in the calculation.

Retention factors, $k$, for seven TCAs were determined with four methanol/20 m*M* phosphate buffer mobile phases covering the composition range of 75/25 to 90/10 methanol/buffer v/v% (0.57 to 0.80 mole fraction of methanol) at pH 7 and 9. Similar experiments were undertaken for mobile phases at pH 3 covering the composition range of 0.308 to 0.640 mole fraction methanol. The variation of $\log k$ with mole fraction of methanol was linear for all three mobile phase pH values studied. The $S'$ and $C$ values for mobile phase of different pH resulting from the linear regression are listed in Table 2. Linear fits resulted in an average $r^2$ value of 0.997 under all conditions. As expected, $S'$ and $C$ values for pH 7 and 9 showed only small differences, because further increasing the pH above 7 should have minimal effect on the retention. Data at pH 3 also fit the linear relationship well, even though analytes were completely ionized. However, $S'$ and $C$ values for pH 3 were very different from those for pH 7 or 9, because the ionized analytes ($BH^+$) behave differently than their neutral forms (B).

For EFLC experiments, mobile phase compositions were carefully chosen such that the mole fraction of methanol remained constant at 0.534, in order to keep the retention contribution from methanol constant. Experiments were conducted at pH 7. Fig. 3A represents retention variation as a function of the amount of $CHF_3$ added. The variation in log $k$ with mole fraction of $CHF_3$ was not linear but was better fit by a quadratic dependence with an average $r^2$ value of 0.996.

From a more practical point of view, the methanol/$H_2O$ mole ratio was held as constant of 1.78, corresponding to 80/20 volume ratio, while the amount of $CHF_3$ addition was varied to observe the retention variation. Results are shown in Fig. 3B. In both Fig. 3A and B, the effect of $CHF_3$ addition on retention factor became less pronounced as the mole fraction of $CHF_3$ increased.

To further examine the performance under LC and EFLC conditions, chromatographic parameters, including the retention factor, $k$, and asymmetry factor, $A_{10}$ under LC (64/36 mole% methanol/20 m$M$ phosphate buffer) and EFLC (53.4/30.0/16.6 methanol/20 m$M$ phosphate buffer/$CHF_3$) conditions were compared using constant mobile phase velocity for the two conditions. The asymmetry factors were approximately the same for both LC and EFLC, except for TH. Fig. 4 shows the resulting two chromatograms for HPLC and EFLC. The time of analysis for EFLC was markedly reduced because for all solutes the retention factors decreased and the efficiency increased.

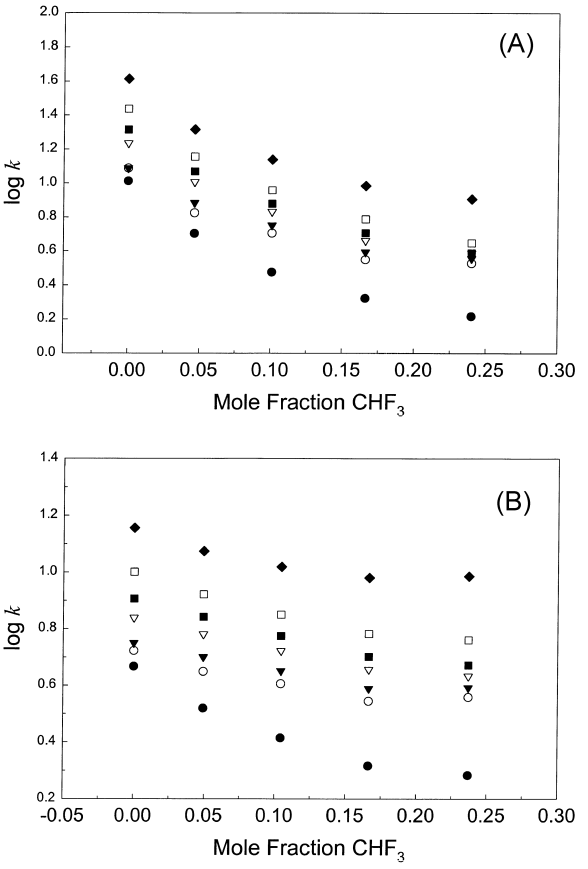



Fig. 3. Plots of log $k$ as a function of mole fraction $CHF_3$ for seven TCAs under EFLC conditions using mobile phases of methanol/20 m$M$ phosphate buffer/$CHF_3$ at pH 7. FL (●), DE (○), NO (▼), IM (▽), AM (■), CL (□), TH (♦). (A) Mole fraction of methanol was held constant at 0.534; (B) methanol/$H_2O$ mole ratio was held constant at 1.78.

Table 2
Comparison of experimentally determined $C$ and $S'$ values for seven TCAs under LC conditions at pH 3, pH 7 and pH 9

| TCA | $C$ | | | $S'$ | | | $r^2$ | | |
|---|---|---|---|---|---|---|---|---|---|
| | pH 3 | pH 7 | pH 9 | pH 3 | pH 7 | pH 9 | pH 3 | pH 7 | pH 9 |
| FL | 3.00 | 2.81 | 2.75 | −5.05 | −3.35 | −3.17 | 0.9949 | 0.9996 | 0.9940 |
| DE | 2.10 | 2.53 | 2.59 | −4.46 | −2.81 | −2.81 | 0.9990 | 0.9988 | 0.9943 |
| NO | 2.56 | 2.52 | 2.62 | −4.65 | −2.75 | −2.88 | 0.9995 | 0.9924 | 0.9917 |
| IM | 7.66 | 2.92 | 2.86 | −4.54 | −3.22 | −3.07 | 0.9990 | 0.9932 | 0.9948 |
| AM | 2.18 | 3.13 | 3.01 | −4.59 | −3.45 | −3.20 | 0.9999 | 0.9993 | 0.9950 |
| CL | 2.51 | 3.38 | 3.25 | −4.56 | −3.70 | −3.43 | 0.9964 | 0.9993 | 0.9953 |
| TH | 2.77 | 3.67 | 3.55 | −4.83 | −3.91 | −3.67 | 0.9924 | 0.9993 | 0.9952 |

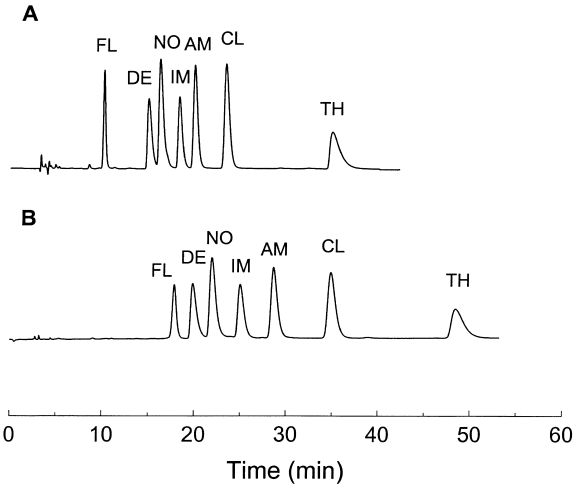



Fig. 4. Chromatograms of separation of seven TCAs at pH 7. (A) EFLC condition: mobile phase of 53.4/30.0/16.6 mole% methanol/20 m*M* phosphate buffer/$CHF_3$; (B) LC condition: mobile phase of 64.0/36.0 mole% methanol/20 m*M* phosphate buffer.

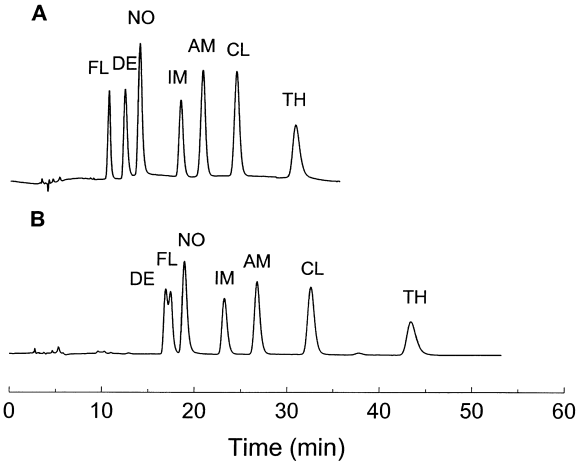



Fig. 5. Chromatograms of separation of seven TCAs at pH 7 with TEA modifier addition. (A) EFLC condition: mobile phase of 53.4/30.0/16.6 mole% methanol/20 m*M* phosphate buffer/$CHF_3$ (12 m*M* TEA); (B) LC condition: mobile phase of 64.0/36.0 mole% methanol/20 m*M* phosphate buffer (12 m*M* TEA).

### *3.4. Effect of addition of TEA as modifier*

Peak shape is also a critical parameter in assessing separation performance, especially for separation of basic analytes. As described above, the peak shapes for many analytes were tailing under both LC and EFLC conditions at mobile phase of pH 7.

For separation of basic analytes, it is also a common practice to add an amine (e.g. 0.1% triethylamine) into the mobile phase. The added amine serves as silanol blocking agent to reduce undesired interactions between basic functionalities and ionized silanol groups, therefore improving chromatographic performance, e.g. better peak shape and higher efficiency.

A 12 m*M* concentration of triethylamine (TEA) was included in aqueous buffers for both LC and EFLC separations. Fig. 5 shows the chromatographic separation with TEA modifier under both LC and HPLC conditions (at constant linear velocity, but not at constant reduced velocity because the diffusion coefficients were not available). In comparison to separations under similar condition without TEA addition, peak asymmetry factors generally improved. However, the introduction of TEA also altered selectivity. Under the LC condition, the selectivities were adversely affected such that FL and DE became partially resolved; while under EFLC condition, baseline separation was still obtained. Even with 25 v/v% $H_2O$ present in the mobile phase, baseline separation under LC conditions with TEA addition still could not be achieved and markedly longer retention times resulted as shown in Fig. 6.

When a comparison of the analysis time was made under LC and EFLC conditions using constant mobile phase linear velocities, EFLC with TEA showed the fastest analysis time, while LC without TEA showed the longest analysis time and interestingly EFLC without TEA and LC with TEA showed similar analysis times (compare chromatograms in Figs. 4 and 5).

### *3.5. Viscosity, pressure drop and on-line mixing*

Previous studies of enhanced-fluidity liquid chromatography have demonstrated that a substantially lower pressure drop was always achieved when a fluidity enhancing agent such as $CO_2$ or $CHF_3$ was added to the liquid mobile phase. The significant decrease in pressure drop is attributed to the de-

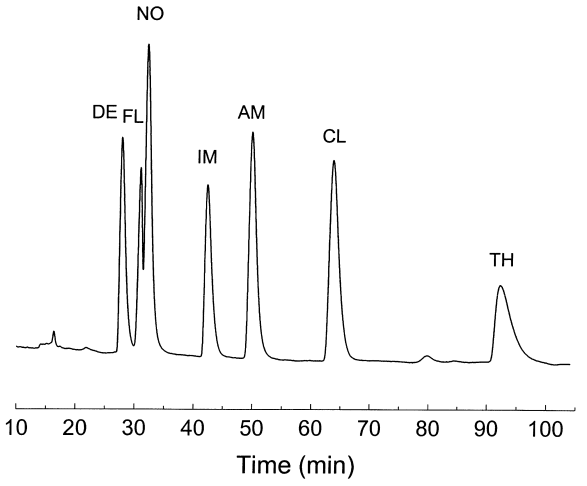



Fig. 6. Chromatogram of LC separation of seven TCAs using mobile phase of 57.2/42.8 mole% methanol/20 m*M* phosphate buffer (pH=7, 12 m*M* TEA).

creased mobile phase viscosity. According to Darcy's law [32], the pressure drop $\Delta P$ ($\Delta P = P_1 - P_2$) is described by Eq. (3):

$$\Delta P = \frac{\phi \eta \mu l}{d_p^2} \quad (3)$$

where $\phi$ is a dimensionless flow resistance parameter; $\eta$ is the viscosity of the mobile phase; $L$ is the column length and $d_p$ is the particle diameter of the packing material. For constant linear velocities, the pressure drop is proportional to the viscosity of the mobile phase. Therefore, under isocratic conditions (constant mobile phase composition) and a constant volumetric flow-rate (thus constant linear velocity), the pressure drop across the column should also hold constant. Because in these enhanced-fluidity experiments $P_2$ (the outlet pressure of the column) was maintained at a constant pressure of 102 atm (1500 p.s.i.). $P_1$ was expected to remain constant, provided the flow-rate and mobile phase composition remain unchanged.

Because the addition of liquified gas, such as $CHF_3$, can significantly reduce the viscosity of the resulting mixture while the pressure drop is proportional to mobile phase viscosity, continuous $P_1$ readings also dictate the variation in mobile phase viscosity and thus composition. This provides a convenient way of checking the mobile phase composition and therefore the effectiveness of on-line mixing, since $P_1$ and $P_2$ were continuously read from the instrument. In this study, inlet pressure $P_1$ was closely monitored throughout the experiments. A pressure variation of less than 2% was always observed for both LC and EFLC conditions, which was comparable to manufacturer's technical specification.

Viscosities of these mixtures were calculated based on Eq. (3), as the viscosity for methanol/$H_2O$ (80/20 v/v) mixture was known of 1.218 cP [13]. As expected, under the same flow conditions, the more $CHF_3$ added to the mobile phase resulted in the lower viscosity and therefore lower pressure drop. An addition of 16.6 mole% of $CHF_3$ decreased the mobile phase viscosity about as much as 42%; while an addition of 23.7 mole% of $CHF_3$ decreased the mobile phase viscosity up to 56%.

## 4. Conclusions

Buffered liquid mobile phases, with and without $CHF_3$ addition were applied to the separation of seven TCAs covering a wide range of polarity. The chromatographic performance with mobile phase conditions of different pH, with and without $CHF_3$ addition and with the addition of TEA modifier was studied. Mobile phase with pH 7 gave the best separation results while raising pH to 9 did not increase their retention nor improve the separation.

The addition of $CHF_3$ not only improved the separation efficiency, but also greatly reduced the retention time, thus resulting in an overall improvement in the separation performance. The addition of $CHF_3$ also provided some selectivity change that might be advantageous in tuning separation performance. In the case of the separation for the TCAs in this study, best separation results were achieved using EFLC mobile phase with TEA addition.

This study also demonstrated that EFLC and LC can be readily achieved on commercial SFC instruments. The easy, precise and accurate control of mobile phase composition and instantaneous on-line mixing provided by the instrument will make EFLC a viable and attractive technique with fast method development and improved speed of analysis in finding more applications.

## Acknowledgements

The authors thank Gilson Inc. for the loan of Gilson SF3 supercritical chromatograph for this study. We also thank the National Science Foundation for financially supporting this project.

## References

[1] Y. Cui, S.V. Olesik, Anal. Chem. 63 (1991) 1812.
[2] Y. Cui, S.V. Olesik, J. Chromatogr. 4691 (1995) 151.
[3] S.T. Lee, S.V. Olesik, Anal. Chem. 66 (1994) 4498.
[4] S.T. Lee, S.V. Olesik, J. Chromatogr. A 707 (1995) 217.
[5] S.T. Lee, S.V. Olesik, S.M. Fields, J. Microcolumn Sep. 7 (1995) 477.
[6] T.S. Reighard, S.T. Lee, S.V. Olesik, Fluid Phase Equilib. 123 (1996) 215.
[7] S.T. Lee, T.S. Reighard, Fluid Phase Equilib. 122 (1996) 223.
[8] H. Yuan, S.V. Olesik, J. Chromatogr. A 785 (1997) 35.
[9] H. Yuan, I. Souvignet, S.V. Olesik, J. Chromatogr. Sci. 35 (1997) 409.
[10] M. Ashraf-Khorassani, L.T. Taylor, P. Zimmerman, Anal. Chem. 62 (1990) 1177.
[11] T.S. Oostdyk, R.L. Grob, J.L. Snyder, M.E. McNally, Anal. Chem. 65 (1993) 596.
[12] D. Wen, S.V. Olesik, Anal. Chem. 72 (2000) 475.
[13] D.R. Lide, H.P.R. Frederikse, CRC Handbook of Chemistry and Physics, CRC, Boca Raton, FL, 1997.
[14] R.C. Downing (Ed.), Fluorocarbon Refrigerants Handbook, Prentice Hall Inc, Englewood Cliffs, New Jersey, 1988, p. 98.
[15] J. Zhao, S.V. Olesik, Fluid Phase Equilib. 154 (1999) 261.
[16] A.R. Ravishankara, A.A. Turnipseed, N.R. Jensen, S. Barone, M. Mills, C.J. Howard, S. Solomon, Science 263 (1994) 71.
[17] H. Yuan, S.V. Olesik, Anal. Chem. 70 (1998) 1595.
[18] A.C. Moffat, J.V. Jackson, M.S. Moss, B. Widdop, Clarke's Isolation and Identification of Drugs, The Pharmaceutical Press, London, UK, 1986.
[19] M. Adamczyk, J.R. Fishpaugh, C.A. Harrington, D.E. Hartter, R.E. Hruska, A.S. Vanderbilt, Ther. Drug Monit. 15 (1993) 436.
[20] L.E. Holister, Drugs 22 (1981) 129.
[21] M. Bogusz, M. Erkens, R.D. Maier, I. Schroder, J. Liq. Chromatogr. 15 (1992) 127.
[22] E. Bosch, P. Bou, H. Allemann, M. Roses, Anal. Chem. 68 (1996) 3651.
[23] A. Bhattacharyya, A.K. Maandal, S.C. Lahiri, Electrochim. Acta 25 (1980) 559.
[24] M. Paabo, R.A. Robinson, R.G. Bates, J. Am. Chem. Soc. 87 (1964) 415.
[25] Cs. Horvath, W. Melander, I. Molnar, Anal. Chem. 49 (1977) 142.
[26] A. Albert, E.P. Serjeant (Eds.), The Determination of Ionization Constants: A Laboratory Manual, Chapman and Hall, New York, 1984, p. 35.
[27] R.J.M. Vervoort, F.A. Mans, H.J. Hindriks, J. Chromatogr. 623 (1992) 207.
[28] D.V. McCalley, J. Chromatogr. A 769 (1997) 169.
[29] Cs. Horvath, W. Melander, I. Molnar, J. Chromatogr. 125 (1976) 129.
[30] C.F. Poole, S.K. Poole (Eds.), Chromatography Today, Elsevier, Amsterdam, The Netherlands, 1991, p. 462.
[31] S.T. Lee, Ph.D. Dissertation, Department of Chemistry, The Ohio State University, Columbus, OH 43210, 1995.
[32] A.E. Scheidegger (Ed.), Introduction to Modern Liquid Chromatography, The MacMillan Company, New York, 1960, p. 68.