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Rational method development strategies on a fluorinated liquid chromatography stationary phase: Mobile phase ion concentration and temperature effects on the separation of ephedrine alkaloids☆

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Abstract

Fluorinated, silica-based stationary phases are becoming increasingly popular alternatives to traditional alkyl phases owing to their differential selectivity and retention for a variety of analyte classes. In this report, the ion-exchange mechanisms characteristic of a fluorinated phase are exploited to rapidly develop separation conditions for ephedrine alkaloids and synephrine using a mobile phase compatible with mass spectrometry. A linear relationship of basic analyte retention with the reciprocal of ammonium acetate concentration is first established. This linear relationship can then be used to optimize retention and selectivity in just two experiments. The relationship of retention with temperature is also explored. Greater retention with increasing temperature is demonstrated on the fluorinated phase at high percentages of organic modifier, which is in contrast to behavior observed in typical reversed-phase separations. The unexpected observation is explicated based on the reduction in solvent solvating power with increasing temperature. As solvation power of the mobile phase decreases, decreased solvation of both mobile phase and ionized surface groups of the stationary phase leads to stronger interactions between analyte and stationary phase. Both mobile phase ion concentration and temperature are shown to be powerful tools for the manipulation of analyte retention and selectivity.

Keywords: Liquid chromatography; Mass spectrometry; Pentafluorophenylpropyl; Stationary phase; Fluorinated; Retention mechanisms; Ion-exchange; Temperature

1. Introduction

Liquid chromatography is most often accomplished on reversed-phase stationary phases based on alkyl-bonded silica particles [1]. Stationary phases manufactured using alternative bonded phases have, however, become increasingly popular due to the differences in selectivity and retention that they often provide. Fluorinated stationary phases, in particular, are gaining acceptance as alternatives to common C18 and C8 phases owing to their unique selectivity [2]. In addition to dispersive interactions available on traditional alkyl phases, the pentafluorophenylpropyl phase also allows for dipole–dipole, pi–pi, charge transfer and ion-exchange interactions [2,3].

Perfluorinated phases have shown unique selectivity in several column classification studies. Neue grouped fluorinated phases separate from C18 and cyanopropyl phases claiming differences in "extended polar selectivity" and "phenolic selectivity [4]." In an investigation of 135 commercially available stationary phases, Euerby noted significant selectivity differences for a set of fluorinated phases [5]. These findings prompted further study in which the authors report orthogonal selectivity of the fluorinated phases as compared

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to both phenyl- and alkyl-based columns [2]. Orthogonal selectivity was especially evident for the retention of basic analytes. Needham reported the exceptional retentivity of a series of tricyclic antidepressants and calcium channel blockers at high percentages of organic modifier on a fluorinated phase, noting the substantial increase in LC–MS response under such conditions [6]. In a recent report, we investigated the molecular interactions contributing to retention on a pentafluorophenylpropyl (PFPP) stationary phase at high organic modifier content [7]. A major conclusion from this study was that retention of protonated bases at high percentages of organic modifier is characterized by strong ion-exchange interactions with ionized surface silanol groups plus additional non-ionic interactions [7].

The objective of the present study was to rapidly develop a method for the separation of several related alkaloids using the knowledge that ionic interactions dominate mechanisms of retention on the PFPP phase. First, a linear relationship of basic analyte retention with the reciprocal of mobile phase ion concentration is established. The linear dependence allows for optimization of mobile phase ion concentration in just two experiments. For demonstration purposes, we chose a set of ephedrine alkaloids as a representative set of polar, basic analytes that are difficult to retain and separate on traditional alkyl stationary phases. Synephrine was included as it is a constituent of currently available herbal dietary supplements indicated for weight loss with structural features similar to the banned ephedrine-based supplements [8]. Several literature methods have been reported for separation of ephedrine alkaloids, however these often call for time-consuming derivatization procedures [9,10], ion-pairing modifiers [11] or strong cation-exchange stationary phases [12]. The latter methods are inappropriate for LC–MS analyses owing to the non-volatility of the mobile phase additives. Gay and White recently reported a system suitable for LC–MS analysis of ephedrine alkaloids, however, the high aqueous content of the mobile phase employed is likely to limit the sensitivity of the method [13]. Although our intent here was not to rigorously develop a method for the analysis of ephedrine and related compounds, suitable separation for the analytes under the high organic conditions on the PFPP phase provides a starting point for further development of a sensitive LC–MS method.

In addition to mobile phase ion concentration, the dependence of retention on temperature at high organic modifier on the PFPP phase was also investigated. Although the dependence of retention on temperature in classical reversed-phase chromatography has been well studied [14–17], temperature effects for systems dominated by ion-exchange retention mechanisms are not well understood. In fact, most of the abnormalities (negative slopes in van't Hoff plots) observed in the literature appear to have some connection to underlying ion-exchange phenomena. The unexpected relationship of analyte retention with temperature observed in this study is discussed in terms of the effects of mobile phase solvating power and its dependence on temperature.

2. Experimental

2.1. Reagents and standards

All compounds chosen for the retention studies were obtained from Sigma (St. Louis, MO, USA). Separate stock solutions of each analyte were prepared by dissolving a weighed amount of each compound in methanol to obtain concentrations of 1 mg/mL. Stock solutions were stored at 0–4 °C when not in use. Samples for analysis were prepared by diluting stock solutions with the respective mobile phase for the study to a final concentration of 100 or 10 μg/mL. All HPLC reagents were obtained from Aldrich (Milwaukee, WI, USA) and were of HPLC grade or better and were used without further purification. HPLC grade water used throughout the study was obtained from a Barnstead Nanopure Diamond™ (Boston, MA, USA) source.

2.2. HPLC columns, conditions and apparatus

Pentafluorophenylpropyl-bonded liquid chromatography columns (Discovery HS F5) were obtained from Supelco (Bellefonte, PA, USA). The columns, packed with 5 μm particles with surface area of 300 m^2/g were either 50 or 150 mm in length and had 4.6 mm internal diameters.

Mobile phases employed in the study were prepared by dissolving ammonium acetate in either 85 or 90% aqueous acetonitrile mixtures to obtain the desired molar concentration of ammonium counter ion. All mobile phases were premixed. The pH values of the mobile phases were unadjusted (pH 6.7 prior to the addition of organic modifier). HPLC–UV analyses were conducted using a Hitachi (San Jose, CA, USA) LaChrom Elite HPLC system equipped with a quaternary pump, autosampler, in-line degassing unit, temperature control unit and photo-diode array UV detector. Acquisitions were made using EZChrom Elite version 3.1.3 from Scientific Software Inc. (Pleasanton, CA, USA). Retention data were acquired in triplicate using 10 μl injections, a flow rate of 1 mL/min and UV detection at either 220 or 215 nm. Temperature was varied within the specifications of the instrument (10–65 °C). System hold-up time (t_0) was estimated by injecting pure methanol. The possibility of retention for traditional t_0 markers such as uracil on the PFPP phase precluded their use. Hold-up times measured in this manner were consistent throughout the studies.

LC–MS data were acquired using a Waters (Milford, MA, USA) 2790 HPLC system equipped with a quaternary pump, autosampler and a Hitachi LaChrom Elite column temperature control module. The HPLC system was connected to a Waters/Micromass ZQ single quadrupole mass spectrometer via an electrospray ionization interface operating in positive ion mode. Retention data were acquired at a flow rate of 1 mL/min and a temperature of 45 °C. The analytes (ephedrine alkaloids and synephrine) were prepared as a mixture in 90% acetonitrile at 10 μg/ml.

3. Results and discussion

3.1. Dependence of retention on mobile phase ion concentration

At high organic mobile phase compositions, the dominant mechanism contributing to retention for cationic analytes on the PFPP phase is ion-exchange [7]. For an ion-exchange process involving singly charged analytes, the dependence of retention on mobile phase counter ion concentration (ammonium in this case) may be expressed as:

$$\log k' = -\log[C^+]_m + \log \beta_{IEX} \quad (1)$$

where $[C^+]_m$ represents the concentration of counter ion in the mobile phase and β_{IEX} is a constant for a given system which incorporates the phase ratio, ϕ, ion-exchange capacity of the stationary phase, $[A^-]_s$, and the ion-exchange equilibrium constant, K_{IEX} [18].

$$\beta = \phi K_{IEX}[A^-]_s \quad (2)$$

The relationship of retention dependence on mobile phase counter ion concentration was investigated by monitoring the retention of amitriptyline (see Fig. 2) on the PFPP phase from 2 to 20 mM ammonium acetate in 85% acetonitrile. As shown in Fig. 1, a linear dependence of retention on mobile phase counter ion is demonstrated by the coefficient of variation of 0.9990. The linear relationship has also been verified using a number of basic analytes under similar conditions on the PFPP phase in our laboratories [data not shown]. Note that the slope of the regression line in Fig. 1 is not equal to −1 as predicted by Eq. (1). A slope of 1 is obtained where ion-exchange is the only mechanism of interaction present. The PFPP phase is thus shown to interact with the solutes via additional mechanisms of retention as reported previously [7].

The linear relationship of retention with mobile phase counter ion concentration allows for facile optimization of this parameter with only two experiments. Retention data

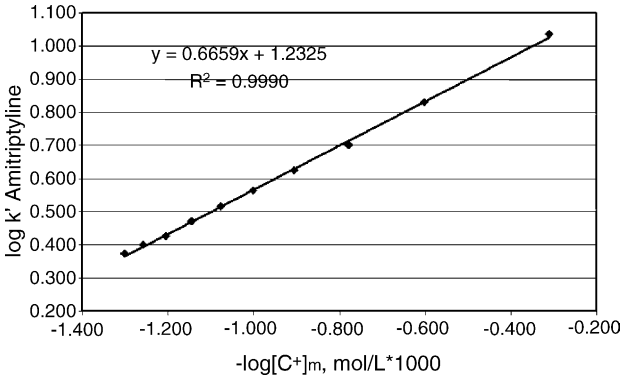

Fig. 1. Dependence of amitriptyline retention on mobile phase ion concentration at 85% acetonitrile using the PFPP stationary phase. Conditions: column: Discovery HS F5 (50 mm × 4.6 mm, 5 μm particle size), mobile phase: ammonium acetate varying in concentration from 2 to 20 mM in 85% acetonitrile, temperature: 35 °C, flow rate: 1 mL/min, detection: UV at 220 nm.

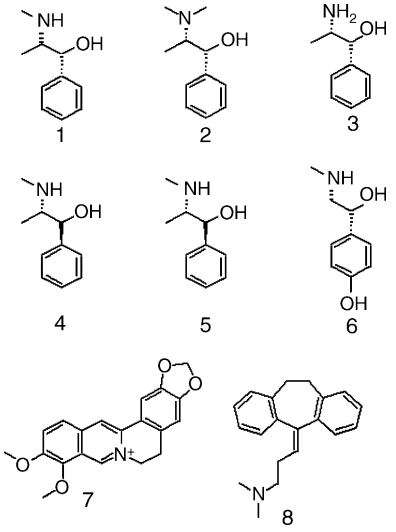

Fig. 2. Structures of ephedrine alkaloids, synephrine and berberine: (1) ephedrine; (2) methylephedrine; (3) norephedrine; (4) pseudoephedrine; (5) norpseudoephedrine; (6) synephrine; (7) berberine and (8) amitriptyline.

for norephedrine, synephrine, methylephedrine, ephedrine, methylpseudoephedrine and pseudoephedrine were acquired at 2 and 10 mM ammonium acetate in 90% acetonitrile on the PFPP phase. The structures of the ephedrine alkaloids along with synephrine are presented in Fig. 2. The compounds are all basic and thus readily interact via ion-exchange mechanisms with the ionized silanol groups on the PFPP phase using mobile phases at high organic percentages. The analytes are also difficult to retain on typical reversed-phase stationary phases because they are ionized at pH values suitable for HPLC analysis. The analytes represent a class of compounds well suited for analysis using fluorinated stationary phases.

The acquired data for the ephedrine alkaloids and synephrine are plotted in Fig. 3 and the respective slope and intercept data are presented in Table 1. Slope values

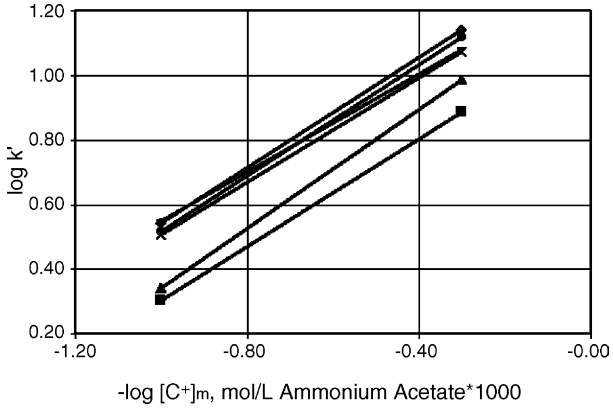

Fig. 3. Retention of norephedrine (■), synephrine (▲), methylephedrine (×), ephedrine (●), pseudoephedrine (♦) and methylpsuedoephedrine (–) as a function of mobile phase ionic concentration on a pentafluorophenylpropyl stationary phase. Mobile phase: 2 or 10 mM ammonium acetate in 90:10 (v/v) acetonitrile:water, flow rate: 1 mL/min, temperature: 35 °C, detection: UV at 215 nm.

Table 1
Slope and intercept data for ephedrine alkaloids and synephrine retention dependence on mobile phase ion concentration

Analyte	Slope	Intercept
Norephedrine	0.84	1.14
Synephrine	0.92	1.27
Methylephedrine	0.81	1.32
Ephedrine	0.86	1.38
Pseudoephedrine	0.86	1.40
Methylpseudoephedrine	0.76	1.31

Conditions: column: pentafluorophenylpropyl stationary phase, mobile phase: 2 or 10 mM ammonium acetate in 90:10 (v/v) acetonitrile:water, flow rate: 1 mL/min, temperature: 35 °C, detection: UV at 215 nm.

approaching 1 demonstrate that the primary mechanism contributing to retention is ion-exchange. Deviation from the value of 1, however, shows that more interactions are taking place in addition to ion-exchange. The intercept value is proportional to the retention of the analytes due to interactions other than ion-exchange assuming all ion-exchange interaction are suppressed at 1 M mobile phase counterion concentration. The data in Table 1 show that the more polar norephedrine and synephrine analytes exhibit relatively low *y*-intercept values as would be expected based on non-ionic mechanisms (low reversed-phase retention of polar solutes). The ephedrine/pseudoephedrine and methylephedrine/methylpseudoephedrine pairs show similar *y*-intercept values as would also be expected from their similar polarities. The magnitude of the *y*-intercept for these two pairs of analytes however, does not correlate with their expected retention in reversed-phase chromatography. This is a significant observation as it suggests that the non-ionic interactions at high organic modifier are dissimilar in behavior to traditional reversed-phase interactions using more aqueous solvents. The different slope and *y*-intercept values obtained for these similar analytes demonstrates the power of mobile phase ion concentration for manipulation of selectivity and retention on the fluorinated phase.

As shown in Fig. 3, the selectivity for all analyte pairs except for ephedrine and pseudoephedrine changes as a function of the mobile phase ionic concentration. Methylephedrine and methylpsuedoephedrine coelute at 2 mM ammonium acetate, however this pair is well resolved at a 10 mM concentration. At 10 mM ammonium acetate the methylephedrine and ephedrine as well as methylpseudoephedrine and psuedoephedrine pairs are unresolved. From the data it is apparent that resolution can be achieved between 3 and 4 mM ammonium acetate. A concentration of 4 mM was chosen as a compromise between resolution and the speed of analysis. In addition to selectivity changes it is readily observed from the data that elution time can also be rapidly optimized using this approach.

3.2. Dependence of retention on temperature

Temperature can be used as a variable to optimize selectivity and run time in chromatographic analyses. The retention dependence on temperature is related to the van't Hoff equation for chromatography [19]:

$$\ln k' = \ln \beta - \frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \quad (3)$$

where retention is linearly related to $1/T(K)$ assuming that the adsorption enthalpy (ΔH°) does not vary with temperature. In initial retention studies for the ephedrine alkaloids performed at 35 and 60 °C (data not shown) differing slopes were observed for the analytes, demonstrating the potential to temperature to serve as a selectivity parameter. To further investigate the relationship of retention with temperature, retention data for norephedrine, synephrine and methylephedrine were acquired from 10 to 45 °C in increments of 5 °C. In addition to these three analytes, a quaternary ammonium compound, berberine, was also included in the study. Berberine (see Fig. 2), due to its permanently charged state, will not change in retention as a function of changes in its degree of ionization and therefore is suitable for probing the ionization state of the surface silanols as a function of temperature [20].

Fig. 4 shows the van't Hoff plots for the four analytes in the study. Berberine is shown to increase in retention in a linear fashion ($R^2 = 0.9621$) from 10 to 45 °C. In reversed-phase separations a decrease in retention with increased temperature typically occurs. Since the dispersive (hydrophobic) mechanism is exothermic and the enthalpy term dominates the Gibbs free energy of the interaction, retention decreases with increasing temperature [21], however, several reports have shown behavior for basic compounds where retention increases with increasing temperature [17]. McCalley reported negative slopes in the van't Hoff plots for nortriptyline and quinine on a base-deactivated C18 stationary phase [22]. The increase in retention with temperature was explicated by a decrease in the degree of ionization of the analytes combined with an increase in effective pH as a function of increasing temperature. The decrease in degree of ionization was said to lead to increased hydrophobic retention and thus a negative slope in the van't Hoff plot. Since the degree of ionization of berberine is unaffected by pH or temperature, the explanation cannot account for the observations of this study. Temperature change may effect changes in pH and pK_a values for the surface silanols, however evidence in the literature suggests that acid dissociation constants for benzoic [23] and phosphoric acids [21,22] are relatively independent of temperature over this range of temperatures. The observed independence of acid pK_a values on temperature is likely a result of a concomitant increase in pH and the acid pK_a value. If the pK_a values of surface silanol groups increase at a greater rate than the rise in pH, the increase in berberine retention could be explained by the enhanced degree of surface silanol ionization. It is assumed here, however, that the acidic silanol groups act in accordance with the acids noted in the literature.

The increase in retention of berberine is best explained by a decrease in solvation energy with increasing temperature. It is well established that solvent solvating power decreases

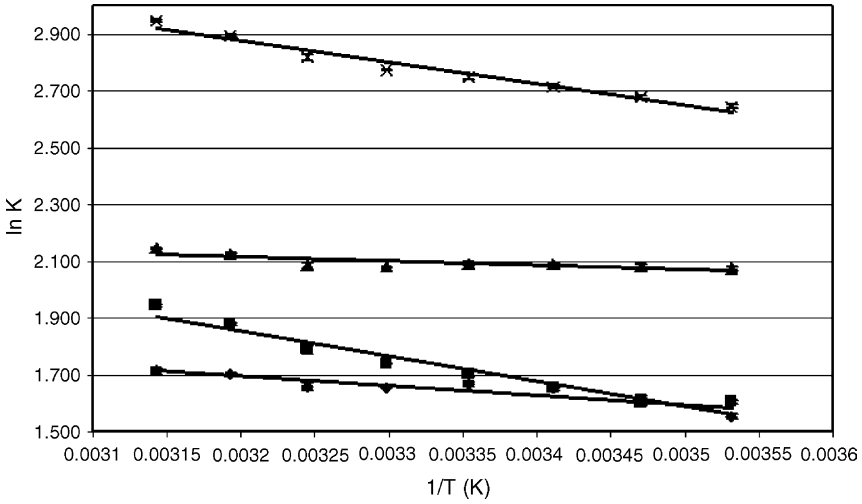

Fig. 4. Retention of berberine (×), synephrine (■), methylephedrine (▲) and norephedrine (♦) as a function of temperature on a pentafluorophenylpropyl stationary phase. Mobile phase: 4 mM ammonium acetate in 90:10 (v/v) acetonitrile:water, flow rate: 1 mL/min, detection: UV at 220 nm.

with increasing temperature. This effect has been demonstrated through the thermosolvatochromism of many spectroscopic probes in various solvent systems [24–28]. Solvating power is based on an empirical scale intended to describe the solute–solute and solute–solvent interactions in addition to contributions to solvation from quantifiable solvent physical parameters such as dielectric constant, dipole moment and polarizability. Because the quantitative parameters represent bulk properties of the solvent, they do not account for specific solvent–solute or solute–solute interactions at the molecular level [24].

A change in solvating power may alter the acid–base equilibrium constants for the solutes as well as the surface silanol groups because of differential effects on solvation of the neutral and ionized forms. As noted by McCalley, if the degree of ionization changes, the dispersive interactions of the solute with both the solvent and the stationary phase change based on reversed-phase chromatographic theory [29]. In addition, and apparently of great importance under the chromatographic conditions of this study, decreased solvation power results in an increase in ionic interactions between cationic solutes and the anionic support. Stronger solvent–solute interactions at low temperatures are more effective at shielding the ions from interacting. As the temperature increases, weaker solvent–solute interactions render the ions more interactive, resulting in an increase in ion-exchange interactions.

The slope close to 1 in the ion concentration study demonstrates that synephrine retention is primarily due to ion-exchange mechanisms. Synephrine exhibits a strong positive response of retention on increasing temperature. Progressively weaker responses are observed for norephedine and methylephedrine retention as a function of temperature. The magnitude of analyte retention response to temperature thus appears to be dependent on the relative importance of ionic and non-ionic contributions. The differential response in analyte retention to temperature for these similar solutes demonstrates that temperature is a powerful tool to manipulate selectivity in this mode of chromatography. This is in contrast to the limited dependence of selectivity on temperature in reversed-phase separations [29].

Optimization of temperature for the separation of the ephedrine alkaloids and synephrine was established at 45 °C. The data in Fig. 4 suggest, by extrapolation to higher temperatures, that the retention of methylephedrine and synephrine would continue to converge. At lower temperatures, the

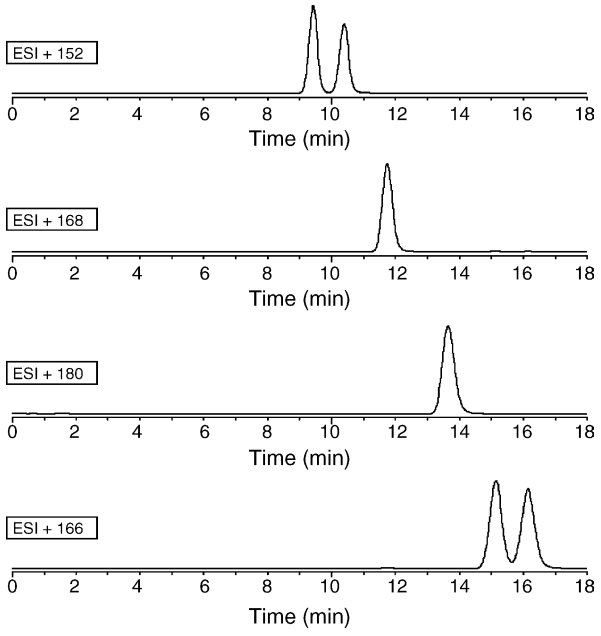

Fig. 5. Separation of: (1) norephedrine; (2) norpseudoephedrine; (3) synephrine; (4) methylephedrine; (5) ephedrine and (6) pseudoephedrine on a pentafluorophenylpropyl stationary phase, 150 mm × 4.6 mm, 5 μm. Mobile phase: 4 mM ammonium acetate in 90:10 (v/v) acetonitrile:water, flow rate: 1 mL/min, temperature: 45 °C, detection: ESI–MS operating in positive ion mode.

retention of synephrine and norephedrine tend toward convergence (see Fig. 5).

4. Conclusions

Strategies for rapid method development can be established through knowledge of the dominant molecular interactions that contribute to retention and selectivity and the parameters that control such interactions. At high percentages of organic modifier, a pentafluorophenylpropyl stationary phase has been shown to retain basic analytes via dominant ion-exchange mechanisms. In this study we have demonstrated that retention and selectivity of basic analytes on a fluorinated stationary phase are strongly dependent on mobile phase ion concentration. Only two experiments are required for optimization of this parameter due to the linear dependence of $\log k'$ on mobile phase ionic concentration. The result is facile method development in terms of both selectivity and run time. In addition, retention due to non-ionic mechanisms in high organic mobile phases is demonstrated to differ from traditional reversed-phase chromatography using more aqueous mobile phases.

Temperature was shown to be an effective parameter for the manipulation of retention and selectivity. Retention at high organic modifier percentages on the fluorinated phase increases with increasing temperature in contrast to chromatographic processes dominated by dispersive interactions (RPLC). This observation was explained by the lower solvation strength of the mobile phase at higher temperatures that consequently results in more loosely solvated ions at higher temperatures. The relatively poor solvation renders the ions more active toward ion-exchange interactions.

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