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Comparison of reversed-phase and hydrophilic interaction liquid chromatography for the separation of ephedrines

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ABSTRACT

The separation of highly basic solutes is an ongoing challenge, especially in achieving suitable retention and peak shapes for compounds such as ephedrines that have both high pK_a values ($\geq$9.3) and low lipophilicity ($\log P \leq 1.74$). In this study we investigate the application of HILIC as a potential alternative approach for the fast separation of the ephedrines phenylpropanolamine, cathine, ephedrine, pseudoephedrine and methylephedrine in doping control analysis. Using sub-2 μm bare silica bridged-ethylene hybrid (BEH) HILIC material, we evaluate the effects of organic modifier, buffer pH and concentration and column temperature on the retention and selectivity of these compounds. Highly symmetrical peak shapes for all ephedrines were achieved under HILIC conditions ($A_{s0.1} \leq 1.1$). We also compare the kinetic performance of the optimised HILIC separation with a previously developed high pH reversed-phase approach. van Deemter curves and kinetic plots for the two approaches are constructed and illustrate the kinetic benefits of HILIC over the reversed-phase approach. Improved mass transfer characteristics and enhanced diffusion with HILIC offers lower C-term coefficients of 1.46 and 5.68 for ephedrine with HILIC and RPLC, respectively.

1. Introduction

Achieving symmetrical peak shapes and acceptable retention factors for hydrophilic basic compounds with high performance liquid chromatography (HPLC) is challenging by traditional reversed-phase approaches. In cases where these types of solutes are also polar, such as ephedrines which are highly basic (pK_a values > 9.3) and hydrophilic ($\log P$ values < 1.74), the challenge is amplified and provokes alternative solutions for separation by LC (see Table 1 for analyte properties). In human sport, the World Anti-Doping Agency (WADA) prohibits the use of many of the ephedrines since they are regarded as stimulants [1]. At present, cathine (norpseudoephedrine), ephedrine, pseudoephedrine and methylephedrine are prohibited in competition above threshold concentrations. To control use, urine samples from athletes must be analysed for their presence and quantitative confirmation analyses performed where necessary [1]. Ephedrine and pseudoephedrine are diastereoisomers, as are their related demethylated substances cathine and phenylpropanolamine. Also related is the compound methylephedrine, differing only by a single methyl group from the ephedrine geometric isomers. Mass spectrometric (MS) detection is necessary for the unequivocal identification of these analytes in doping control, presenting a further challenge since the diastereoisomers share very similar fragmentation patterns, and hence each analyte must be separated chromatographically prior to detection.

Previous methods of separation have included gas chromatography coupled to nitrogen phosphorous detection (GC–NPD) [2] or mass spectrometry (GC–MS) [3], high performance liquid chromatography coupled to ultra-violet detection (HPLC–UV) [4], where GC–MS requires derivatisation and HPLC–UV lacks the sensitivity and selectivity of MS. Hyphenation to mass spectrometry, when dealing with analytes in biological matrices, offers decidedly more information for identification and confirmation of a positive doping suspect. More recently, anti-doping drug analysis methodology is being transferred to LC–MS, offering faster analysis times and simple sample preparation, critical factors where fast turnaround times are required, as well as increased sensitivity [5–8]. However, certain approaches to achieving good retention and peak shape for basic compounds with reversed-phase (RP) LC are not amenable to MS detection, such as ion-pairing reagents and high ionic strength buffers. The impact of utilising perfluorinated ion-pairing reagents for LC–MS analysis of a range of basic drugs was evaluated by Schubert and Oberacher [9] noting that under overloading conditions simple carboxylic acid additives provide much inferior peak shapes and poor retention for hydrophilic analytes

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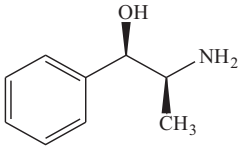
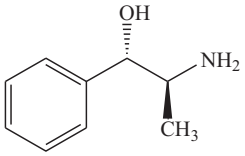
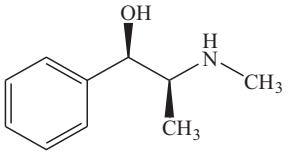
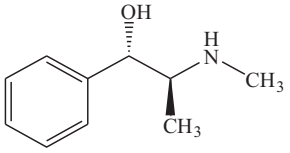
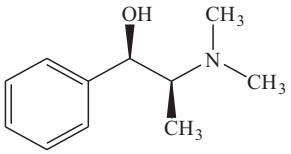
Table 1
Properties of the compounds considered in the present study.

Compound	Structure	pK_a [a]	log P [b]	log D (pH 5) [c]	log D (pH 10) [c]
Phenylpropanolamine (norephedrine)		9.4	0.81	−3.59	0.71
Cathine (norpseudoephedrine)		9.4	0.81	−3.59	0.71
Ephedrine		9.6	1.05	−3.55	0.90
Pseudoephedrine		9.8	1.05	−3.75	0.84
Methylephedrine		9.3	1.74	−2.56	1.66

[a] pK_a values obtained from Clarke's Analysis of Drugs and Poisons.
[b] log P values obtained from ChemSpider.
[c] log D values calculated from pK_a and log P values using the equation: $\log D = \log P + \log[1/(1 + 10^{pK_a - pH})]$.

such as nicotine. The use of fluorinated ion-pairing reagents were shown to significantly improve peak shape, however this was at the detriment of mass spectrometric performance. Fleiger [10] investigated the use of perfluorinated ion-pairing reagents as applied to the reversed-phase separation of β-blockers reporting useful increases in retention and improved peak shapes when compared to acetic acid. Stuppner and co-workers [11] utilised the ion-pairing reagent sodium dodecyl sulfate (SDS) for the separation of ephedra alkaloids in dietary supplements. Alternative solutions involve the use of highly aqueous mobile phases in order to retain these polar analytes [12,13] but such approaches are associated with poor MS desolvation and stationary phase de-wetting. Other strategies such as the use of chaotropic salts and ionic liquids [14,15] as mobile phase additives for the analysis of hydrophilic amines have been shown to yield highly symmetrical and efficient separations, however such approaches are unsuitable for interfacing to electrospray ionisation mass spectrometry. Suitable retention has also previously been achieved by manipulating the mobile phase so as to reduce the degree of analyte ionisation, recently made possible with high pH stable stationary phases [16], or by utilising complementary stationary phase materials such as the pentafluorophenyl variant [17,18].

Hydrophilic interaction chromatography (HILIC) has recently become a widespread alternative to RPLC for achieving good retention and peak shapes for polar or ionisable analytes, and is also amenable to MS detection [19–21]. In addition, HILIC offers particular advantages over RPLC approaches, including lower back pressures and enhanced desolvation with electrospray ionisation (ESI) owing to the large percentage of organic modifier in the mobile phase. The resulting lower back pressures permit the use of faster flow rates for increased sample throughput, longer columns for enhanced resolution or use of sub-2 μm particle materials with traditional pumping systems, while improved desolvation with ESI mass spectrometry offers better sensitivity and lower limits of detection. Nevertheless, the technique also brings with it certain disadvantages over RPLC, in particular regarding the complex and poorly understood retention mechanisms. Unlike the well-identified mechanisms of retention in RPLC, there are several proposed mechanisms of interaction at play in HILIC [22–24], complicating the predication of retention which is widely valued in RPLC. Nor does HILIC offer the flexibility and applicability that RPLC does, with only limited tools available to manipulate a separation, and is associated with longer re-equilibration times and problems with sample solubility.

However, where other methods cannot provide appropriate retention or peak symmetry, HILIC has been shown to be a powerful alternative for separating polar, hydrophilic compounds. Here we investigate the suitability of HILIC as an alternative to RPLC for the fast chromatographic separation of ephedrine isomers. The compounds in question are structurally similar, and without the presence of a shape selective moiety bonded to the stationary phase, as is the case with bare silica, the separation of these solutes may prove difficult. We investigate the effects chromatographic parameters including the proportion of acetonitrile, buffer pH and concentration and column temperature on the HILIC separation in order to determine whether HILIC is a viable option for the retention

and separation of the distinct ephedrines. With these parameters optimised, the same bridged-ethylene hybrid (BEH) material functionalised with C18 ligands is used as a comparator technique to evaluate the benefits of HILIC over RPLC. A kinetic performance comparison is presented between RPLC and HILIC as two possible approaches for the separation of ephedrines.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (HPLC grade), ammonium bicarbonate, ammonium hydroxide solution (35%) and formic acid (98%) were obtained from Fisher Scientific (Loughborough, UK). Ammonium acetate, ammonium formate, uracil and toluene were purchased from Sigma (Poole, UK). Norephedrine, norpseudoephedrine, ephedrine, pseudoephedrine and methylephedrine were purchased as hydrochloride salts from Sigma (Poole, UK). Water was purified by an ultra-pure water system (Millipore, UK).

2.2. Solutions

2.2.1. Reversed-phase mobile phase

Ammonium bicarbonate buffer was prepared at 10 mM in purified water and adjusted to pH 10 with ammonium hydroxide solution (35%). The mobile phase was pre-mixed at a composition of 90:10 (v/v) 10 mM ammonium bicarbonate pH 10:CH_3CN by adding 100 mL of acetonitrile to 900 mL 10 mM ammonium bicarbonate pH 10.

2.2.2. HILIC mobile phase

Ammonium formate (pH 3 and 4) and ammonium acetate (pH 5) buffers were prepared separately at 200 mM in purified water and adjusted to the appropriate pH with formic acid (98%). Typically, the mobile phase preparation consisted of 95:5 (v/v) CH_3CN:200 mM buffer prepared by taking 50 mL of the 200 mM stock buffer and adding to 950 mL acetonitrile. The pre-mixed mobile phase was then sonicated to ensure complete dissolution of the buffer salt.

2.2.3. Sample preparation

Stock solutions were prepared at a concentration of 1 mg/mL for norephedrine, norpseudoephedrine, ephedrine and methylephedrine and pseudoephedrine in methanol at −20 °C until required. Standard working solutions at 50 μg/mL were prepared by diluting stock solutions with the appropriate mobile phase for either HILIC or reversed-phase operations.

2.3. LC conditions

Liquid chromatography was carried out on a Waters Acquity UPLC® (Waters Corp., Milford, USA) which consisted of a sample manager, binary solvent delivery system and a PDA detector. The autosampler was thermostatted at 6 °C. All injections were made in duplicate and averaged for all experiments. For the van't Hoff experiments a Waters Xevo QTof-MS (Waters Corp., Manchester, UK) was used to track the compounds of interest. The columns were connected to the injection valve using the temperature stabiliser tubing so as to ensure that adequate eluent pre-conditioning took place. For the kinetic performance experiments sample injections of 1 μL were made using a 2 μL loop in the partial loop with needle overfill mode and for the mass spectrometry experiments 5 μL injections were made using a 5 μL loop in full loop mode. Empower 2 or MassLynx V4.1 software (Waters Corp., Milford, USA) was used for data acquisition.

2.3.1. Reversed-phase conditions

Separations were carried out on an Acquity UPLC® system (Waters Corp., Milford, USA) with an Acquity BEH C_{18} 1.7 μm, 2.1 mm × 50 mm column for the reversed-phase separation equipped with a 0.2 μm in-line filter. Isocratic chromatography was performed using a prefixed mobile phase of 90:10 (v/v) 10 mM ammonium bicarbonate pH 10:CH_3CN. The flow rate was 500 μL/min and column temperature set at 60 °C. The weak and strong needle wash lines of the Acquity UPLC® system were placed in 90:10 H_2O:CH_3OH (v/v) and 10:90 H_2O:CH_3OH (v/v) respectively. The elution conditions were slightly modified from a previously reported separation of ephedrines by Gray et al. [16] except performed using sub-2 μm BEH C_{18}.

2.3.2. HILIC conditions

Acquity BEH HILIC 1.7 μm, 2.1 × 50 or 100 mm columns were used for the HILIC separation using a 0.2 μm in-line filter. The mobile phase comprised of premixed 95:5 (v/v) CH_3CN:200 mM buffer for the HILIC separation. Flow rate was 500 μL/min and column temperature set at 50 °C unless otherwise specified. The weak and strong needle wash lines of the Acquity UPLC system were placed in 95:5 (v/v) CH_3CN:H_2O and 50:50 (v/v) CH_3CN:H_2O, respectively, so as not to cause any interference with the sample plug.

2.4. Detection

Analyte detection was performed using an Acquity PDA detector or a Xevo QToF mass spectrometer (Waters Corp., Milford, USA) equipped with an electrospray ionisation (ESI) source operated in positive ion mode. UV data was collected at 210 nm using a sampling rate of 40 Hz. Columns were connected to either the mass spectrometer ion source or PDA-UV detector using minimal lengths of 0.004 in. ID PEEK tubing.

The MS was operated in MS^E mode collecting two channels of data throughout the run, one with low collision energy (4 V) and one high (ramp from 10 to 20 V) in order to obtain both the precursor and product ions. Source conditions were optimised for each mobile phase composition and for reversed-phase separation the capillary voltage was set at 1.0 kV, the sample cone 10 V, source temperature 120 °C, desolvation temperature 400 °C. In HILIC the capillary voltage was set at 3.0 kV, sampling cone 20 V, source temperature was 120 °C, desolvation temperature 350 °C. For both LC setups the cone gas flow was set at 10 L/h and the desolvation gas was set at 800 L/h. The micro-channel plate detector was operated at 2150 V. Data were collected in centroid mode over an m/z range of 100–1000 Da with a scan time of 0.05 s. Leucine enkephalin (5 ng/mL) was used as the accurate mass reference material, infused through the LockSpray probe at 5 μL/min and acquired every 20 s with a 0.3 s scan time scans (3 scans were averaged).

2.5. Methodology for the construction of van Deemter plots

The kinetic study was performed using phenylpropanolamine, ephedrine and methylephedrine as the test solutes. Column efficiency (N_{col}) was determined from peak widths at half height for the HILIC study. The asymmetry factor (A_s) and peak widths ($w_{0.1}$) were determined at 10% of the peak height for the reversed-phase study. All reported data were obtained after correction for extra-column band broadening (σ^2_{sys}) caused by the instrument, determined by removing the column and replacing it with a zero volume connector

and injecting each solute individually in the relative mobile phase for each study.

$$N_{col} = \frac{(t_{R,total} - t_{R,sys})^2}{\sigma^2_{total} - \sigma^2_{sys}} \quad (1)$$

Data was transformed into on-column plate height as follows:

$$H_{col} = \frac{L}{N_{col}} \quad (2)$$

where L is the column length (m) and H_{col} is plate height (μm).

Plate height data were fitted to the van Deemter equation:

$$H_{col} = A + \frac{B}{u_0} + Cu_0 \quad (3)$$

where A, B and C are the Eddy diffusion, longitudinal dispersion and resistance to mass transfer coefficients respectively, and u_0 is the mobile phase linear velocity, determined in cm/s in this study. SigmaPlot (version 11.0, Systat Software Inc.) software was used to fit the (u_0, H) data to Eq. (3).

2.6. Methodology for the construction of kinetic plots

Based on experimental van Deemter data (u_0, H_{col}) and column permeability values (K_{vO}), kinetic plots were constructed to better visualise the potential of the two analytical systems investigated, accounting for mobile phase flow rate, chromatographic efficiency, generated back pressure and column geometry. Two equations are necessary to transform this experimental data into extrapolated plots of analysis time *versus* efficiency, as outlined by Desmet et al. [25,26]:

$$N = \frac{\Delta P_{\max}}{\eta}\left(\frac{K_{vO}}{\mu_0 H_{col}}\right)_{\text{experimental}} \quad (4)$$

$$t_0 = \frac{\Delta P_{\max}}{\eta}\left(\frac{K_{vO}}{\mu_0^2}\right)_{\text{experimental}} \quad (5)$$

where $\Delta P_{\max}$ is the maximum allowed pressure drop with any given support material, pending also the constraints of the chromatographic apparatus, and η is the mobile phase viscosity (cP). Data was processed and curve fittings made using the Kinetic Plot Creator 3.1 (Vrije Universiteit Brussel, Belgium) for generation of kinetic plots.

3. Results and discussion

3.1. Effect of CH_3CN composition on retention of phenylpropanolamine, ephedrine and methylephedrine

Retention in HILIC is achieved through the establishment of a water rich layer immobilised on the surface of the polar stationary phase, thereby facilitating a multitude of chemical interactions. Upon increasing the concentration of acetonitrile in the mobile phase relative to the aqueous component, an increase in retention is generally observed. As illustrated by McCalley [19], it is essential to include buffer at the appropriate concentration when using HILIC as poor peak shapes are observed if only weak acids are used as mobile phase additives. It is therefore essential to employ a buffer which is soluble in the acetonitrile rich mobile phase. Ammonium acetate and ammonium formate were chosen as suitable buffers in this study, which have the added benefit of being MS compatible. Also of importance is the concentration of the buffer as long equilibration will be necessary if low concentrations are used, whereas they will precipitate in the organic rich mobile phase at high concentrations and contamination within the LC–MS ion source may occur causing signal suppression. In the first instance an overall concentration of 10 mM was employed, and it was noted that higher concentrations resulted in a precipitate being formed when mixed with acetonitrile. Initially, prior to attempting the separation of the geometric isomers, retention of the individual amines was established using 10 mM ammonium acetate buffer adjusted to pH 5. As observed in Fig. 1, there was an increase in retention with increasing acetonitrile concentration for phenylpropanolamine, ephedrine and methylephedrine. Although retention was adequate at 90% acetonitrile ($k \sim 2$) for all compounds, little or no separation was achieved between the three compounds. However, by increasing the acetonitrile content further, to 95%, the separation of phenylpropanolamine, ephedrine and methylephedrine was achieved.

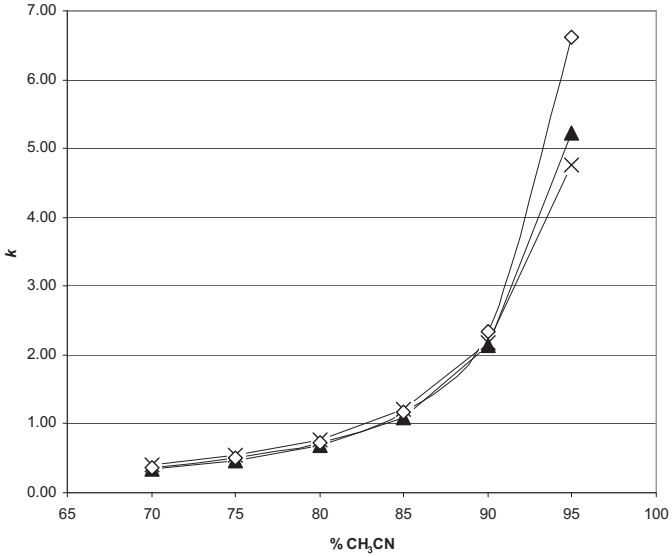


Fig. 1. HILIC retention behaviour (k) of methylephedrine (crosses), phenylpropanolamine (triangles) and ephedrine (diamonds) at 50 °C, constant 10 mM ammonium acetate pH 5.0, as a function of percentage acetonitrile. Column was 10 cm × 2.1 mm ID, HILIC BEH 1.7 μm operated at 500 μL/min.

3.2. Dependence on retention of mobile phase pH

Residual silanols on the surface of silica are reported to have a pK_a of 7.1 ± 0.5 [27], although the bridged-ethylene hybrid (BEH) material used here has been reported to be less acidic than conventional silicas due to surface deactivation [28,29]. According to Neue et al. [29], the pK_a of hybrid packings decreases with increasing organic modifier concentrations by as much as 0.63 units per 30% increase in acetonitrile content. As already shown by Grumbach et al. [30] and McCalley [19], this further highlights the importance of pH as a variable in developing separations, especially where retention is poor and selectivity must be maximised in order to separate structurally similar compounds. In order to investigate the effect of pH so as to improve the retention of the ephedrines, pH 3.0 and 4.0 using 10 mM ammonium formate and pH 5.0 using 10 mM ammonium acetate, measured in the aqueous phase were used. As shown in Fig. 2, a marked change in the selectivity of these analytes is noted with increasing pH. This may be explained by the dissociation of the silanol groups allowing for cation exchange contributions to take place with the charged basic solutes. The strongest retention of the secondary (ephedrine and pseudoephedrine) and tertiary (methylephedrine) amines was encountered at pH 5. Interestingly, the primary amines (phenylpropanolamine and cathine) slightly decreased in retention with increasing pH, which may be the result of interplay between the pK_a of the analytes and dissociation of silanol groups. It appears overall that the resolution of the ephedrine isomers is in fact improved with increasing pH on bare silica, where dissociation of silanols is clearly

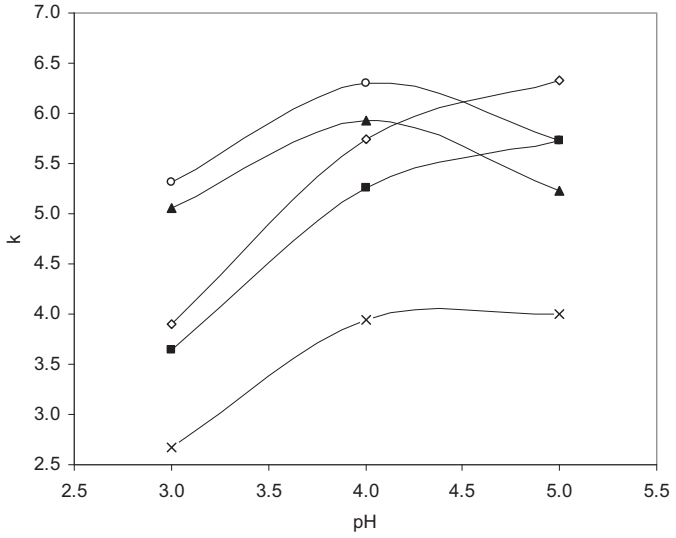


Fig. 2. Retention factors (k) of ephedrines on a 5 cm BEH HILIC column as a function of pH at 50 °C in CH_3CN:200 mM buffer (95:5, v/v). Ephedrine (squares), phenylpropanolamine (triangles), cathine (circles), methylephedrine (crosses), and pseudoephedrine (diamonds).

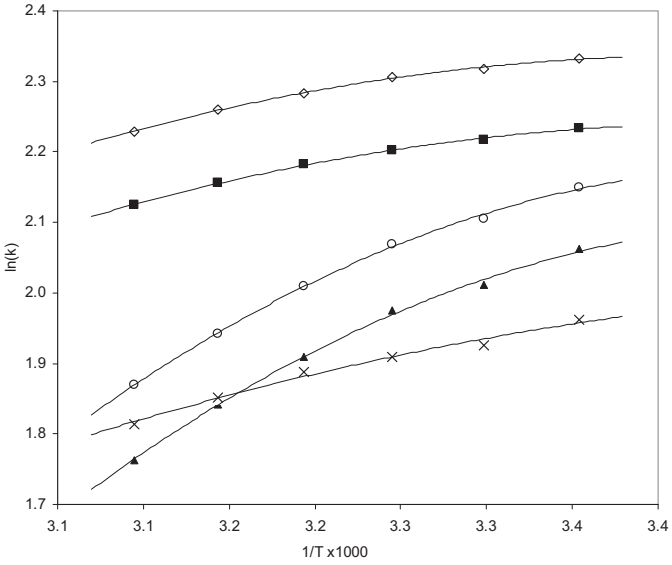


Fig. 4. Plot of log k *versus* $1/T$ using acetonitrile:100 mM ammonium acetate pH 5.0 (95:5, v/v). Compound identities as in Fig. 2.

contributing to selectivity. Although, as seen in Fig. 2, the resolution of the diastereoisomeric pairs is enhanced with the change in selectivity, the co-elution of cathine and ephedrine is apparent. This was subsequently overcome by increasing the column length from 5 cm to 10 cm, offering separation of all five ephedrines. A higher pH eluent was not used since there are no MS compatible buffers in the pH region 5.5–7.0, and pH values above this may incur dissolution of the bare silica stationary phase, particularly at elevated temperatures.

3.3. Effect of increasing temperature on retention

Utilising temperature as a means of manipulating selectivity is a parameter often overlooked in method development. In this study we investigated the effect of temperature, ranging from 25 to 50 °C, on the retention of each analyte with the underivatised BEH silica, represented as log k *versus* $1/T$ (Fig. 3). As shown in Fig. 3, the relationship between increasing temperature and retention is non-linear and a quadratic model was required for the van't Hoff plot. Interestingly, the secondary and tertiary amines experienced little change in retention as a function of temperature at pH 5, whereas the primary amines showed a decrease in retention enthalpy with increasing temperature. At 25 °C the diastereoisomeric pairs (phenylpropanolamine–cathine and ephedrine–pseudoephedrine) are resolved, however the pairs co-elute and inadequate resolution of all ephedrines is observed. As temperature is increased the primary amines lose retention, affording separation of the five ephedrines. This change in selectivity as a function of temperature between the primary amines *versus* the secondary and tertiary amines was shown as a useful means for achieving separation and further highlights the importance of temperature as a variable for achieving resolution.

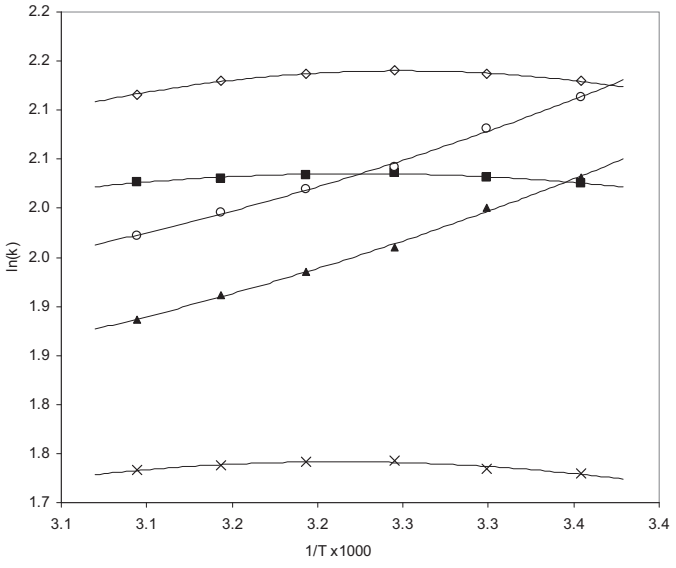


Fig. 3. Plot of log k *versus* $1/T$ using acetonitrile:200 mM ammonium acetate pH 5.0 (95:5, v/v). Compound identities as in Fig. 2.

After observing the unusual retention behaviour as a function of increasing temperature with 10 mM ammonium acetate for secondary and tertiary amines, we investigated the effect of lowering the buffer concentration to 5 mM to allow more exposure of the free silanol groups for cation exchange. An increase in retention was observed for all solutes upon decreasing the buffer concentration, which was expected due to the stronger cation exchange between the basic amines and the silanol groups. However, upon increasing the temperature, a more pronounced decrease in retention was observed for all solutes, Fig. 4. Again, there was a curvature of the retention van't Hoff isotherm. This implies that the enthalpy of retention is dependent on buffer concentration, and that analyte solvation is affected by increasing temperature with higher buffer concentrations. The effect of analyte solvation on retention enthalpy as a function of temperature was recently postulated by Wilson and co-workers [31] for the separation of nucleoside triphosphates. Upon increasing temperature, Buckenmaier et al. [32] showed pK_a decreases for a group of basic solutes, resulting in a decrease in %BH+ for benzylamine of 29%. Further work by Buckenmaier et al. [33] investigated the influence of acetonitrile content on pK_a, showing a decrease of 0.77 units for benzylamine on going from pure water to 60% organic fraction. Thus, there are consequently multiple features at play which are affecting the pK_a values of the ephedrine analytes, exemplified by the curvature of van't Hoff plots suggesting mixed-mode retention. Bidlingmeyer and Henderson [34] postulated that, at lower temperatures, electrostatic and adsorptive forces dominate whereas, at elevated temperatures,

the former is weakened. They also indicated that the amount of adsorbed solvent on the silica surface may also be changing thereby affecting the immobilised phase-ratio found with HILIC.

3.4. Performance comparison between reversed-phase and HILIC

Typically, to retain and separate ephedrine-type compounds by RPLC highly aqueous mobile phases are required, which may yield unfavorable desolvation properties and poor peak shapes making peak integration difficult. This also entails the use of comparatively high viscosity mobile phases in relation to those encountered in HILIC, and as such separation speed is restricted by the high back-pressures generated, especially with the use of sub-2 μm particles. As a comparative method, a RPLC approach modified from a previously reported one [16], which utilised a high pH mobile phase and supra-2 μm C18 BEH particles, was used.

3.4.1. Peak shape comparison between HILIC and reversed-phase approach

The analysis of basic solutes has been of significant interest not only academically but also in the pharmaceutical arena where many drug molecules contain amine groups, as has been recently reviewed [21]. Achieving suitable peak shapes for these molecules requires careful consideration of the chromatographic conditions, particularly where solvent systems must be compatible with MS detection. In order to investigate the column performance obtained with either HILIC or reversed-phase, we constructed van Deemter curves for each system generated using phenylpropanolamine, ephedrine and methylephedrine as test solutes. In the case of the reversed-phase separation, peak asymmetry was problematic, even at the increased temperature (60 °C) and high pH (10.0) employed. In order to ascertain an estimate of the true column efficiency, the Dorsey–Foley procedure [35] was applied where necessary to take into account the peak asymmetry when calculating performance for the reversed-phase study:

$$N_{df} = \frac{41.7(t_r/w_{0.1})^2}{A_s + 1.25} \quad (6)$$

Phenylpropanolamine and ephedrine both yield skewed peak shapes under RP conditions as a function of flow rate and peak asymmetry measured at 10% peak height, as illustrated in Fig. 5. The peak shapes for methylephedrine were found to be similar to those found with HILIC for the flow study, possibly due to the high retention factor ($k \sim 22$) obscuring peak symmetry issues due to on column band broadening. Another possibility is the steric effect of the protecting methyl groups surrounding the tertiary amino group, affording fewer secondary interactions with acidic silanol groups as well as the combined effect of high pH and elevated temperature used here on the analyte pK_a. With increasing flow rate, the peak asymmetry of phenylpropanolamine was found to deteriorate rapidly, with severe peak fronting observed, while for ephedrine, peak tailing was reduced and excellent peak symmetry was achieved. This finding of improved peak asymmetry with increasing flow rate for ephedrine resembles that observed by McCalley [36] for quinine and nortriptyline at intermediate pH. Peak fronting for oxycodone was investigated by Ornaf et al. [37] explaining that the formation of interconverting solvent adducts were involved. However, in their study, this was a temperature dependent effect. As referred to by McCalley [38], based on the work of Neue and co-workers [28] another possibility could be a partial exclusion effect taking place in the stationary phase, which is the result of residual cationic sites that remain from silica manufacture resulting in analyte repulsion. This effect is known to be more pronounced in mobile phases of lower ionic strength. Although, in their study, this was not observed for hybrid-silica packings.

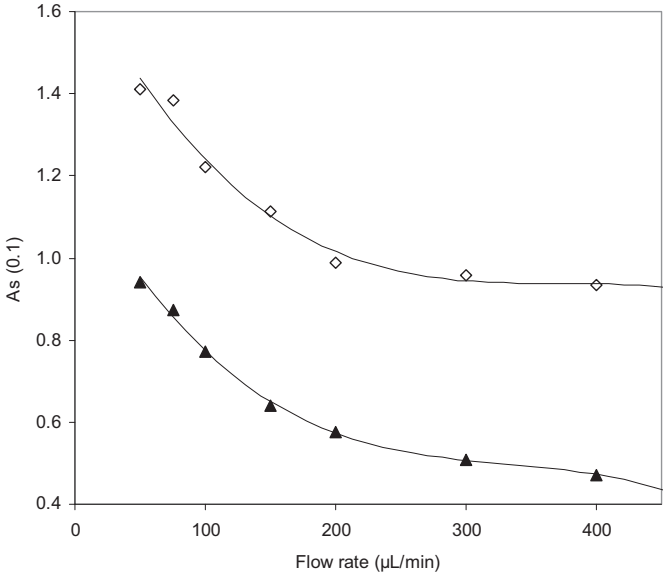


Fig. 5. Effect of increasing flow rate on peak asymmetry (measured at 10% peak height) for ephedrine and phenylpropanolamine using reversed-phase conditions. Compound identities as in Fig. 2.

3.4.2. van Deemter flow study to compare HILIC and reversed-phase

The mobile phase composition used for the reversed-phase approach was mainly aqueous, with 10% CH_3CN compared to the 95% used with HILIC. It has been suggested previously by Walters et al. [39] that working with highly aqueous mobile phases, below the composition used here, can cause de-wetting of C18 stationary phases resulting in retention losses. Due to the hydrophilicity of the ephedrine alkaloids, this approach was essential for adequate retention and a high pH mobile phase was also necessary to improve peak shape and resolution as reported previously [16]. In contrast, with the HILIC retention mechanism, superior peak shapes and performance for all analytes were observed. As seen in Fig. 6(a), the deterioration in column performance for phenylpropanolamine is inherently linked to the effect of flow rate on peak asymmetry and hence poor efficiency (H_{min} = 12 μm) was observed for this analyte. The low log P and log D values (Table 1) for this compound, in comparison to the secondary and tertiary amino containing solutes, can somewhat explain the poor mass transfer properties observed. This low efficiency is not due to the effect of system dead volume as the solute k is 3.5 and losses in performance were calculated to be only 1–2% when correcting for extra-column band broadening. For ephedrine and methylephedrine the benefits of using sub-2 μm stationary phases are apparent due to the C-term flattening of their respective van Deemter curves. We applied the Dorsey–Foley correction to the former due to peak tailing at low flow rate, as alluded to previously. Fig. 6(b), in comparison, shows the van Deemter curve for phenylpropanolamine, ephedrine and methylephedrine under HILIC conditions, where similar column performance is achieved for each compound, approximately 200,000 plates/m. Table 2 shows the van Deemter coefficients from curve fitting for the RPLC and HILIC approaches. As stated elsewhere [40], the retention factor has an impact on the B-term and therefore this coefficient cannot be fairly compared unless similar retention factors are obtained on the two chromatographic systems due to the impact of viscosity and therefore solute diffusivity. Larger B-terms were found for the reversed-phase conditions in all cases, and it is noteworthy that there is a 10 °C difference in column temperature between the evaluations which also contributes to the aforementioned longitudinal band broadening. Clearly, the C-term

Table 2
van Deemter coefficients determined for HILIC and reversed-phase conditions.

Analyte	k	A	B	C	u_{opt} (cm/s)	$HETP_{min}$ (μm)
HILIC (95% CH_3CN)						
Phenylpropanolamine	5.6	3.7	0.47	0.02	0.4	4.7
Ephedrine	6.9	3.2	0.57	1.46	0.6	4.9
Methylephedrine	5.1	2.6	0.59	2.19	0.4	4.7
Reversed-phase (10% CH_3CN)						
Phenylpropanolamine	3.5	5.3	0.54	20.2	0.1	11.8
Ephedrine	8.1	3.0	0.91	5.68	0.5	7.4
Methylephedrine	22.6	3.6	1.02	0.91	0.5	5.7

for phenylpropanolamine under RPLC conditions was much larger than that under HILIC conditions. The fairest comparison for *C*-term values would be the relative performances of ephedrine since the retention factors are similar on both systems, found to be 1.46 for HILIC and 5.68 for reversed-phase. Overall, *A*-term coefficients were in the range of 2.5–5 for both columns, with the exception of phenylpropanolamine, suggesting similar packing qualities.

3.4.3. Kinetic plots of HILIC and reversed-phase

As shown in Table 3, the viscosity of the organic rich mobile phase used in HILIC, 0.32 cP, was far lower than that of the reversed-phase eluent, 0.51 cP. This affords faster analyte diffusivity in the mobile phase, enhancing solute mass transfer and thereby preserving column efficiency when higher flow rates are desired for high throughput analyses. For this reason, the kinetic benefits of the HILIC approach are unparalleled, since instrument pressure limitations restrict high flow rates in RPLC due to the large back pressures generated, a function of packing particle diameter, column length and bed permeability. The latter is denoted by:

$$K_{vO} = \frac{\mu_0 \eta L}{\Delta P_{col}} \quad (7)$$

where μ_0 is the linear velocity (m/s), η is viscosity (cP), L is column length (m) and ΔP_{col} is the system corrected pressure drop (Pa). Using eluents of lower viscosity affords the chromatographer to use longer columns than those limited by higher viscosity operations resulting in enhanced resolution, or faster flow rates for reduced analysis time. This concept was shown by McCalley [41] using conventional pressures (280 bar) with long columns (45 cm) packed with 2.7 μm superficially porous particles yielding 100,000 plates with analysis times between 7.5 and 15 min. Using the approach of Desmet et al. [25,26] we constructed kinetic plots based on the experimental data generated for the van Deemter flow study.

Fig. 7(a) and (b) represents kinetic plots for phenylpropanolamine, ephedrine and methylephedrine generated under HILIC and reversed-phase conditions. As shown in Fig. 7(b), significant gains in plate number *versus* analysis time can be realised using HILIC conditions. For example, at a t_0 value of 100 s approximately 100,000 theoretical plates per meter are generated, whereas the corresponding reversed-phase system generates around 20,000 for phenylpropanolamine or 30,000 for ephedrine and methylephedrine. This clearly demonstrates the kinetic benefits of performing HILIC for this solute type, and is further emphasised in Fig. 7(a) which fundamentally represents a plot of separation impedance *versus* theoretical plate number. The contrast in separation performance is essentially a function of eluent viscosity, dictated by column permeability, since not only are higher plate numbers achievable using HILIC, but this can be performed at lower pressures using equivalent particle sizes. It is therefore possible to accommodate sub-2 μm materials in the HILIC mode using conventional instruments, however the advantages would only be preserved using well optimised equipment as previously highlighted [42,43]. Column permeability using the HILIC conditions was found to be double that of reversed-phase, reflected in the 50% lower flow resistance, shown in Table 3. Minimum separation impedances (E) for ephedrines under HILIC conditions were found to be <5000 for the three probe solutes, compared to 12,000–50,000 for RPLC yielding significantly lower plate numbers.

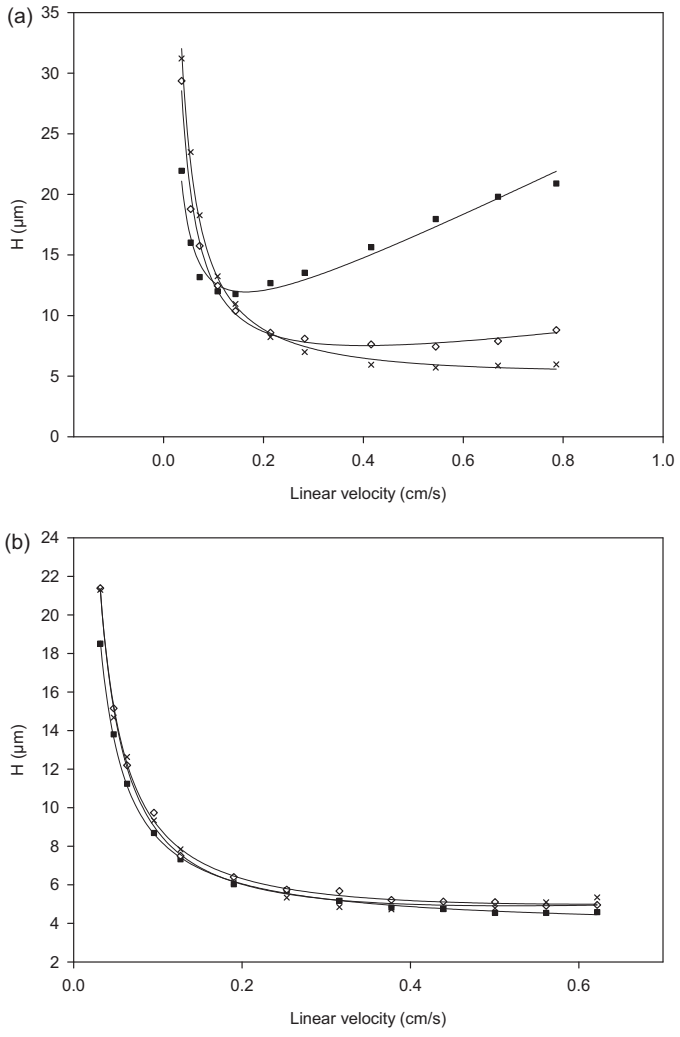


Fig. 6. van Deemter curves for reversed-phase (a) and HILIC (b) phenylpropanolamine (squares), ephedrine (diamonds) and methylephedrine (crosses).

Table 3
Kinetic plot parameters determined for HILIC and reversed-phase systems. Viscosity (η) was calculated using the Chen–Horvath correlation [44].

Kinetic parameter	HILIC (50 °C)	Reversed-phase (60 °C)
Permeability, K_{vo} (m^2)	5.50E−15	2.83E−15
Viscosity, η (cP)	0.318	0.506
Flow resistance, Φ	526	1020
Max. ΔP (bar)	564	702

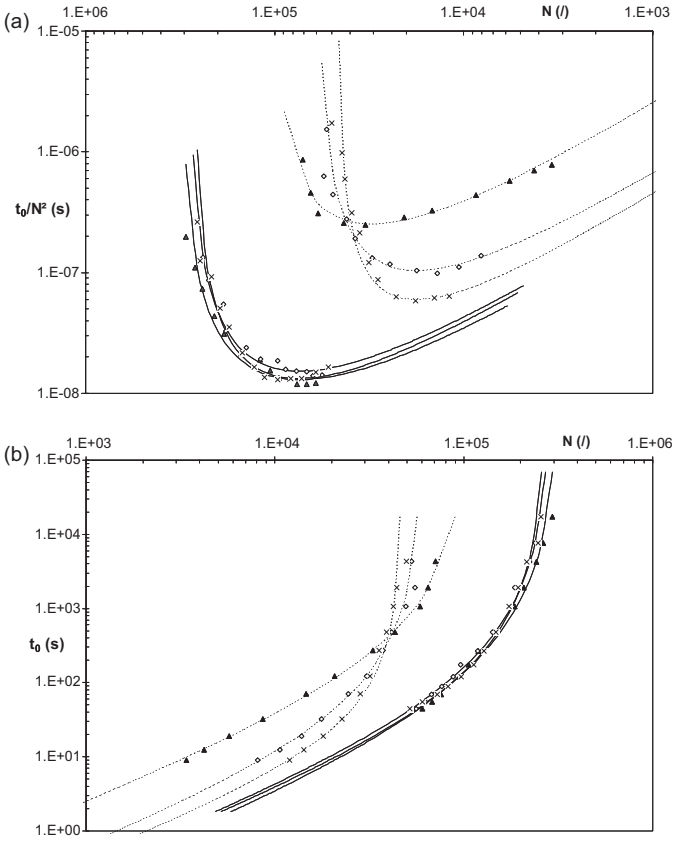


Fig. 7. Kinetic plot representation of pressure drop limited plate number based on van Deemter data were (a) impedance and (b) are plate time dependent. Dotted and full lines represent the reversed-phase and HILIC systems, respectively. Data points are as in Fig. 6.

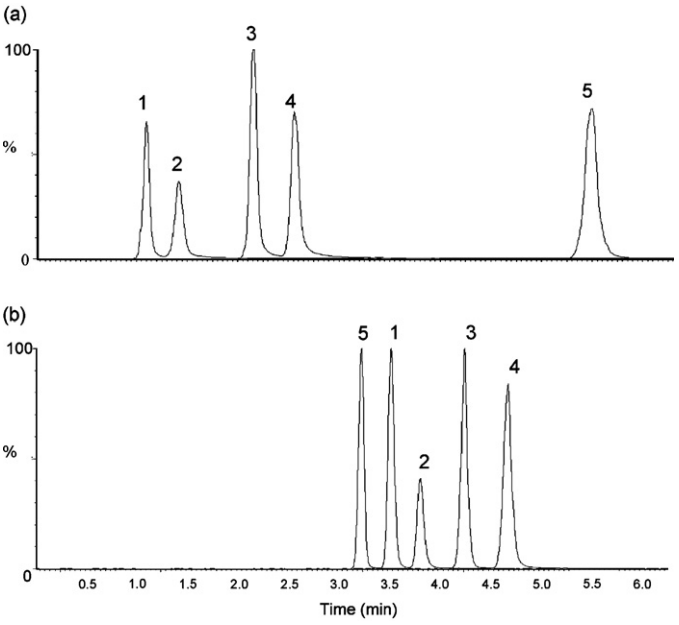


Fig. 8. Chromatograms illustrating the separation of phenylpropanolamine (1), cathine (2), ephedrine (3), pseudoephedrine (4) and methylephedrine (5) under the reversed-phase conditions as in Section 2.3.1 (a) and HILIC conditions (b). HILIC was performed using 95:5 (v/v) CH_3CN:200 mM ammonium acetate pH 5, 100 mm × 2.1 mm ID Acquity BEH HILIC 1.7 μm, column temperature 50 °C. Flow rate for both separations was 500 μL/min.

3.5. Application: analysis of ephedrines by LC–QTof-MS

The final separation of structurally related hydrophilic ephedrine solutes is shown in Fig. 8 as by the overlaid extracted ion chromatograms obtained by interfacing with a fast scanning QTof mass spectrometer. Our separation compares very favorably to previously reported methodologies for the analysis of ephedrines, using mobile phase eluents and flow rates (typically ≤ 500 μL/min) amenable to mass spectrometry. The five compounds can be separated by HILIC in under 5 min with high column efficiency (~20,000 plates on column) and excellent peak shapes ($A_{s0.1} \leq 1.1$) with WADA acceptable retention factors (Table 2).

4. Conclusions

We have demonstrated the advantages of HILIC as an attractive alternative to RPLC for the separation of hydrophilic ephedrines. Our study highlights the ability of pH and temperature to manipulate the separation of critical pairs, particularly where analytes are structurally similar. Superior peak shapes are obtained under HILIC conditions compared to the tailing peaks inherently observed with basic compounds under reversed-phase conditions. The HILIC separation also affords significantly lower mobile phase viscosity and higher solute diffusivity allowing yet faster separations without the expense of column performance.

We fully intend in later studies to compare a quantitative method between the two techniques encompassing typical validation parameters, since it is proposed that HILIC will provide enhanced sensitivity owing to improved desolvation of the organic rich mobile phase with ESI. This additional advantage will be of particular significance for analytes where sensitivity is a problem and lower limits of detection are required, and may also permit the use of small sample volumes and reduced sample preparation efforts, such as direct dilution and injection of a sample. The HILIC approach must also be assessed for repeatability and robustness to ensure suitability as a routine technique. This would then allow for full comparison of the technologies evaluated herein to be realised.

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References

[1] World Anti-Doping Agency, Montreal, Canada, available: http://www.wada-ama.org, 2011.
[2] P. Van Eenoo, F.T. Delbeke, K. Roels, P. De Backer, J. Chromatogr. B: Biomed. Sci. Appl. 760 (2001) 255.
[3] M.H. Spyridaki, C.J. Tsitsimpikou, P.A. Siskos, C.G. Georgakopoulos, J. Chromatogr. B: Biomed. Sci. Appl. 758 (2001) 311.
[4] P.J. van der Merwe, L.W. Brown, S.E. Hendrikz, J. Chromatogr. B: Biomed. Appl. 661 (1994) 357.
[5] M. Kolmonen, A. Leinonen, A. Pelander, I. Ojanperä, Anal. Chim. Acta 585 (2007) 94.
[6] M.H. Spyridaki, P. Kiousi, A. Vonaparti, P. Valavani, V. Zonaras, M. Zahariou, E. Sianos, G. Tsoupras, C. Georgakopoulos, Anal. Chim. Acta 573–574 (2006) 242.
[7] J.-O. Thörngren, F. Östervall, M. Garle, J. Mass Spectrom. 43 (2008) 980.
[8] M. Mazzarino, X. de la Torre, F. Botrè, N. Gray, D. Cowan, Drug Test. Anal. 2 (2010) 311.
[9] B. Schubert, H. Oberacher, J. Chromatogr. A 1218 (2011) 3413.
[10] J. Flieger, J. Chromatogr. A 1217 (2010) 540.
[11] M. Ganzera, C. Lanser, H. Stuppner, Talanta 66 (2005) 889.
[12] K. Deventer, O.J. Pozo, P. Van Eenoo, F.T. Delbeke, J. Chromatogr. B 877 (2009) 369.
[13] F. Badoud, E. Grata, L. Perrenoud, M. Saugy, S. Rudaz, J.L. Veuthey, J. Chromatogr. A 1217 (2010) 4109.
[14] J. Flieger, A. Czajkowska-Żelazko, J. Sep. Sci. 34 (2011) 733.
[15] L. Pan, R. LoBrutto, Y.V. Kazakevich, R. Thompson, J. Chromatogr. A 1049 (2004) 63.
[16] N. Gray, A. Musenga, D.A. Cowan, R. Plumb, N.W. Smith, J. Chromatogr. A 1218 (2011) 2098.

[17] D.S. Bell, H.M. Cramer, A.D. Jones, J. Chromatogr. A 1095 (2005) 113.
[18] F. Pellati, S. Benvenuti, J. Pharm. Biomed. 48 (2008) 254.
[19] D.V. McCalley, J. Chromatogr. A 1171 (2007) 46.
[20] Z. Aturki, G. D'Orazio, A. Rocco, K. Si-Ahmed, S. Fanali, Anal. Chim. Acta 685 (2011) 103.
[21] D.V. McCalley, J. Chromatogr. A 1217 (2010) 858.
[22] P. Hemström, K. Irgum, J. Sep. Sci. 29 (2006) 1784.
[23] D.V. McCalley, U.D. Neue, J. Chromatogr. A 1192 (2008) 225.
[24] A.E. Karatapanis, Y.C. Fiamegos, C.D. Stalikas, J. Chromatogr. A 1218 (2011) 2871.
[25] G. Desmet, D. Clicq, P. Gzil, Anal. Chem. 77 (2005) 4058.
[26] G. Desmet, D. Clicq, D.T. Nguyen, D. Guillarme, S. Rudaz, J.L. Veuthey, N. Vervoort, G. Torok, D. Cabooter, P. Gzil, Anal. Chem. 78 (2006) 2150.
[27] J. Nawrocki, J. Chromatogr. A 779 (1997) 29.
[28] A. Mendez, E. Bosch, M. Roses, U.D. Neue, J. Chromatogr. A 986 (2003) 33.
[29] U.D. Neue, C.H. Phoebe, K. Tran, Y.F. Cheng, Z. Lu, J. Chromatogr. A 925 (2001) 49.
[30] E.S. Grumbach, D.M. Diehl, U.D. Neue, J. Sep. Sci. 31 (2008) 1511.
[31] E. Johnsen, S.R. Wilson, I. Odsbu, A. Krapp, H. Malerod, K. Skarstad, E. Lundanes, J. Chromatogr. A 1218 (2011) 5981.
[32] S.M. Buckenmaier, D.V. McCalley, M.R. Euerby, J. Chromatogr. A 1060 (2004) 117.
[33] S.M. Buckenmaier, D.V. McCalley, M.R. Euerby, J. Chromatogr. A 1004 (2003) 71.
[34] B.A. Bidlingmeyer, J. Henderson, J. Chromatogr. A 1060 (2004) 187.
[35] J.P. Foley, J.G. Dorsey, Anal. Chem. 55 (1983) 730.
[36] D.V. McCalley, J. Chromatogr. A 902 (2000) 311.
[37] K. Broglé, R.M. Ornaf, D. Wu, P.J. Palermo, J. Pharm. Biomed. 19 (1999) 669.
[38] D.V. McCalley, J. Chromatogr. A 987 (2003) 17.
[39] T.H. Walter, P. Iraneta, M. Capparella, J. Chromatogr. A 1075 (2005) 177.
[40] R.W. Stout, J.J. DeStefano, L.R. Snyder, J. Chromatogr. A 282 (1983) 263.
[41] D.V. McCalley, J. Chromatogr. A 1193 (2008) 85.
[42] D.V. McCalley, J. Chromatogr. A 1217 (2010) 4561.
[43] K.J. Fountain, U.D. Neue, E.S. Grumbach, D.M. Diehl, J. Chromatogr. A 1216 (2009) 5979.
[44] H. Chen, C. Horvath, Anal. Methods Instrum. (1993) 213.