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Article

Retention Studies for Large Volume Injection of Aromatic Solvents on Phenyl-Silica Based Stationary Phase in RP-LC

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The use of a large volume injection of hydrophobic solvents as diluents for less hydrophobic solutes has already been proven for C18 and C8 stationary phases in reversed-phase liquid chromatography. The same possibility is investigated for a phenyl-hexyl stationary phase using aromatic solvents (benzene, toluene, ethylbenzene and propylbenzene) as diluents for several model analytes also containing aromatic rings. Both hydrophobic interaction and $\pi-\pi$ stacking account for the competitive interaction of both the diluent and model analytes with the phenyl-hexyl phase. A linear decrease in analyte retention factor was observed with an increase of injection volume in the range of 1–100 μL. A moderate peak efficiency decrease was also observed, but peaks of model analytes remained undistorted with minimum band broadening up to 100 μL injection volume. A very small retention decrease was observed when changing the sample diluent in the homologous series: benzene, toluene, ethylbenzene and propylbenzene. The critical conditions for a successful large volume injection of analytes dissolved in studied hydrophobic solvents are for the analyte to have lower hydrophobicity and for the specified solutes to have proper solubility.

Introduction

Generally, when injecting moderate to large sample volumes in reversed-phase liquid chromatography (RP-LC), the diluents should be compatible with and weaker than the mobile phase (1, 2). Ideally, sample solvents should be water or the mobile phase itself. Otherwise, phenomena might occur like band broadening, fronting, tailing or other peak distortions (3, 4). Often, poor solubility of various non-polar analytes in aqueous or polar organic solvents requires the use of hydrophobic solvents for dissolution. Additionally, poor chemical stability in the aqueous media of certain molecules requires the use of aprotic and non-polar solvents (5) as sample diluents in RP-LC. Sensitivity requirements for trace analysis with common spectrometric detection in LC are often addressed by increasing the injection volume of samples from liquid–liquid extraction (LLE). Common solvents like benzene, toluene or hexane, highly non-polar in nature, are routinely used in LLE sample preparation. LLE generally requires evaporation of the extracting solvent, followed by re-dissolution of the dry residue in a solvent compatible with the RP-LC mobile phase. These steps increase the duration of the sample preparation procedure and are prone to inducing errors in the analytical quantitation. Several recent studies showed the possibility of injecting large volumes of hydrophobic solvents in RP-LC that are not compatible with the mobile phase without significant negative effects on retention, efficiency, width or symmetry of analyte peaks (6, 7). This possibility generates both increased sensitivity of the LC quantitation and increased throughput and accuracy of LLE procedures, due to elimination of the steps for solvent evaporation and dry residue re-dissolution (8).

Water-immiscible hydrophobic solvents like alkanes (*n*-hexane, *n*-heptane and *n*-octane), aliphatic alcohols (1-octanol) (7, 9) or more common LLE solvents (ethyl acetate, isopropyl acetate, methyl isobutyl ketone and methyl *tert*-butyl ether) (5, 10) have successfully been employed as sample diluents for direct high or moderate volume injection in RP-LC.

Injection of samples dissolved in these organic solvents is related to their hydrophobicity, which is higher than that of the analytes. In such conditions, the hydrophobic solvent is readily adsorbed in the column head immediately after injection due to its strong stationary phase affinity and low mobile phase solubility. The analyte diffuses out of the solvent plug into the mobile phase and is distributed between the mobile and stationary phases according to its hydrophobicity. Due to the large amount of solvent adsorbed in the stationary phase, parts of the adsorption centers in the stationary phase are no longer available for the analytes, leading to a certain decrease in their retention without an obvious negative effect on peak shape. As previous studies showed (7, 9, 11), several conditions must be accomplished to inject high volumes of such hydrophobic solvents without significant peak shape problems for the target analytes: (i) the solvent should be more hydrophobic than the separated analytes; (ii) the solvent should have a low solubility in the mobile phase so that after injection, it will be strongly adsorbed in the column head; (iii) analytes should have proper solubility in the solvent.

Retention studies concerning large volume injection of sample solvents more hydrophobic than the RP-LC mobile phase have been performed using classical C18 or C8 stationary phases (5–9). Phenyl stationary phases are typically used to bring more selectivity to separations of aromatic compounds, which cannot be fully separated using C18 or C8 phases due to closed values of hydrophobicity (12–15). No literature data was found for large volume injection of hydrophobic aromatic solvents using phenyl-based stationary phases. The aim of this paper was to study the possibility of injecting large volumes of aromatic solvents, used as diluents for several aromatic model analytes, using a phenyl-hexyl stationary phase in an RP-LC mechanism. Both types of interactions between aromatic solvent, aromatic model analyte and aromatic stationary phase

are assumed, namely hydrophobic interactions and π−π stacking (16–18). The effects of increasing the injected volume of hydrophobic solvent in the stationary phase or increasing the chain length of the solvent on the retention factor and peak shape efficiency of the model analytes are also investigated.

Experimental

Chemicals and materials

High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile, and pro-analysis grade benzene, toluene, ethylbenzene and propylbenzene were acquired from Sigma-Aldrich (Steinheim, Germany). Phosphoric acid (pro-analysis grade) was from Merck (Darmstadt, Germany) and triethylamine (pro-analysis grade) was from Fluka (Buchs, Switzerland). Water for chromatography was obtained within the laboratory by means of a TKA Lab HP 6UV/UF instrument. Reference standards of acetylsalicylic acid, benzoic acid, codeine (7,8-didehydro-4,5a-epoxy-3-methoxy-17-methylmorphinan-6a-ol) and trimetazidine hydrochloride [1-(2,3,4-trimethoxybenzyl)piperazine dihydrochloride)], were obtained from European Pharmacopoeia (Strasbourg, France). Phenol, benzyl alcohol and 4,4′-dipyridine pro-analysis grade were obtained from Merck.

Chromatographic system and retention studies

Experiments were performed using an Agilent 1100 series LC system (Agilent, Waldbronn, Germany) consisting of a degasser (G1379A), binary pump (G1312A), autosampler (G1313A), column thermostat (G1316A) and variable wavelength detector (G1314A). Data acquisition and analysis were performed with Agilent Chemstation software, revision B.03.02.

All chromatographic runs were carried out on a single Luna 5u Phenyl-Hexyl 150 × 4.6 mm, 5 μm column, and a Luna C8 with the same dimensions, from Phenomenex. Column temperature was kept at 25°C. All experiments were performed using isocratic conditions and a constant flow rate of 1.0 mL/min. Detection wavelength, mobile phase composition and nature, and the injected amount for each studied solute are given in Table I. Solute retention studies with the injection volume (1–100 μL) and nature of the sample diluent were performed. Several model analytes were tested: phenol, acetylsalicylic acid, benzoic acid, benzyl alcohol, codeine, trimetazidine and 4,4'-dipyridine. The diluents used to dissolve all model analyte compounds was the homologous series: benzene, toluene, ethylbenzene and propylbenzene. Concentrated stock solutions of the model analytes were prepared in methanol, followed by large dilutions in the aromatic solvents. The methanol content in the final solutions was less than or equal to 1%. The injection volume range was between 1 and 100 μL. The analyte samples in hydrophobic aromatic solvents were injected at 1, 5, 10, 20, 50, 75 and 100 μL. To highlight the changes in analyte retention, peak shape, symmetry or efficiency when injected from the previously mentioned hydrophobic solvents, a reference injection of 0.1 μL of the specified model analytes was performed in methanol for comparison. Regardless of the injected volume, the absolute amount of the analyte loaded in the chromatographic column (Table I) was kept constant by modifying its concentration accordingly.

Table I
Mobile Phase Composition, Detection Wavelength and Injection Amount for the Studied Analytes

Model analyte	Wavelength (nm)	Aqueous component of mobile phase	Aqueous–organic ratio (CH_3OH)	Aqueous–organic ratio (CH_3CN)	Injected amount (ng)
Phenol	254	Water	60:40	—	1,000
Acetylsalicylic acid	230	0.2% H_3PO_4	55:45	—	200
Benzoic acid	230	0.2% H_3PO_4	55:45	—	160
Benzyl alcohol	254	Water	65:35	—	5,000
Codeine	237	0.2% TEA pH 6 (H_3PO_4)	—	85:15	500
Trimetazidine	237	0.2% TEA pH 6 (H_3PO_4)	—	85:15	1,000
4,4′-Dipyridine	237	0.2% TEA pH 6 (H_3PO_4)	—	77:23	100

Other retention studies were conducted by changing both the mobile phase organic modifier content and injection volume for phenol, acetylsalicylic acid and benzoic acid. Ranges of 30–50% CH_3OH for phenol and 40–50% CH_3OH for acetylsalicylic and benzoic acids were covered when these solutes were injected from all four studied aromatic diluents (1–100 μL). After each injection of the aromatic solvent, the chromatographic column was washed for 10 min using 100% CH_3CN or CH_3OH, and then the column was re-equilibrated to the initial elution conditions. The fetention factor was calculated with the known formula $k = (t_r - t_0)/t_0$, where t_r is the retention time value for the analyte, and t_0 is dead time, measured by means of the negative peak in the chromatogram.

Results and Discussion

Retention experiments conducted by injection of different volumes of aromatic diluents (benzene, toluene, ethylbenzene and propylbenzene) on the phenyl-hexyl silica-based stationary phase showed that the retention factor (k) of the studied analytes (discussed previously) can be influenced by the injected volume of the diluent. The observed effect was the decrease of k when the volume of diluent was increased. An example of such effect is shown the chromatograms (Figure 1) for two of the studied compounds (acetylsalicylic acid and benzoic acid) injected in propylbenzene diluent in the range of 1–100 μL, while the amount of the analyte injected into the column was kept constant. Similar decreases were observed for the other studied compounds (phenol, codeine, trimetazidine, benzyl alcohol and 4,4'-dipyridine) without significant peak distortion in the chromatograms.

The experimental data of k for all model analytes were then represented as a function of the injection volume (V_{inj}): $k =$ intercept + slope V_{inj}. Very good linear correlations were obtained for all analytes dissolved in all solvents, with high correlation coefficients ($R^2 > 0.99$), except for trimetazidine in ethylbenzene and propylbenzene, for which R^2 values were higher than 0.97. Correlation coefficient values for the studied dependences are given in Table II. These dependences show that k decreases slowly with the increase of the injected volume of samples, and that this decrease ranged between 5 and 12% for the studied compounds. The ratio intercept/slope gives the extrapolated value for the injection volume (V_S)

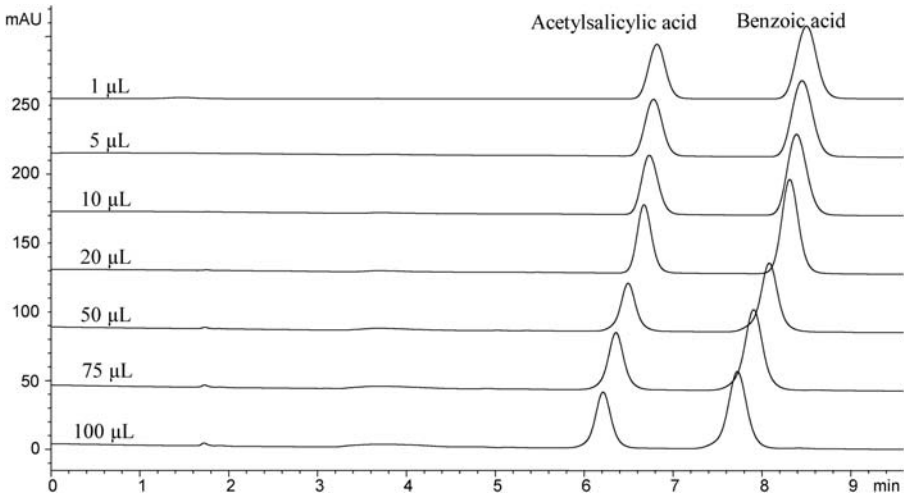

Figure 1. Overlaid chromatograms for acetylsalicylic acid and benzoic acid injected from propylbenzene solutions in the range of 1–100 µL. The mobile phase organic content is 45% CH_3OH. Other chromatographic conditions are given in Table I. A small peak fronting effect is observed for both analytes for injection volumes of 50 µL or higher.

Table II

Slope, intercept and correlation coefficients of the linear regressions obtained for retention factor versus injection volume plots presented in Figure 2*

Model analyte	R^2 / slope values							
	Benzene		Toluene		Ethylbenzene		Propylbenzene	
Phenol	0.9993	−0.0039	0.9993	−0.0039	0.9984	−0.0037	0.9932	−0.0039
Acetylsalicylic acid	0.9965	−0.0035	0.9990	−0.0038	0.9994	−0.0039	0.9967	−0.0040
Benzoic acid	0.9946	−0.0044	0.9991	−0.0050	0.9995	−0.0048	0.9961	−0.0051
Benzyl alcohol	0.9992	−0.0052	0.9997	−0.0051	0.9991	−0.0051	0.9991	−0.0052
Codeine	0.9983	−0.0027	0.9976	−0.0026	0.9993	−0.0027	0.9900	−0.0026
Trimetazidine	0.9910	−0.0048	0.9948	−0.0046	0.9760	−0.0044	0.9838	−0.0037
4,4′-Dipyridine	0.9959	−0.0011	0.9967	−0.0015	0.9940	−0.0016	0.9969	−0.0016
Model analyte	Intercept / −(intercept/slope) values							
	Benzene		Toluene		Ethylbenzene		Propylbenzene	
Phenol	3.782	970	3.779	969	3.660	989	3.665	940
Acetylsalicylic acid	3.551	1015	3.621	953	3.529	905	3.537	884
Benzoic acid	4.672	1062	4.761	952	4.641	967	4.655	913
Benzyl alcohol	4.632	891	4.622	906	4.623	906	4.615	888
Codeine	2.859	1059	2.768	1,065	2.797	1036	2.821	1,085
Trimetazidine	4.016	837	4.049	880	4.100	932	3.979	1,075
4,4′-Dipyridine	2.416	2196	2.426	1,617	2.418	1,511	2.406	1,504

*Note: The value of the extrapolated injection volume for which the analyte is no longer retained in the stationary phase is also given ($V_S = -$intercept/slope).

when the retention factor is 0 (analyte elutes at the dead time, t_0). According to the calculated values from Table II, these values are very high at 1,000 µL. Similar studies conducted on similar C8 or C18 columns showed lower values of this extrapolated parameter (up to 500 µL) (7, 9). This difference can be explained by the possible adsorption of more than one solvent molecule on the bonded chains from stationary phase used in this study, or by a larger degree of derivatization of the silica support for the column used in this study.

The number of theoretical plates for all seven model analytes decreased when the injection volume increased to 100 µL. A decrease of 20 to 50% in peak efficiency was measured, depending on the model analyte. This is because the retention decreases simultaneously with the peak width increase when the injected solvent volume increases. The peak width increase can be explained by the decrease in the number of available adsorption centers for the analyte, and hence a reduced number of adsorption–desorption equilibria. The analyte molecules travel through a part of the column, suffering only longitudinal diffusion and increased peak broadening. Good peak shape for the model analytes was generally observed in the studied range of injection volumes. Depending on the analyte, no, small or moderate peak distortions were observed at injection volumes higher than 50 µL. Peak fronting was the predominant distortion effect, the cause of which was probably the weak solubilization in the mobile phase of some of the hydrophobic solvent molecules. The solubilized solvent molecules move towards the analyte molecules and disrupt the adsorption–desorption equilibria of the latter, generating the analyte peak fronting effect (5, 6). The peak shape and retention decrease with the increase of the injection volume for acetylsalicylic acid and benzoic acid are shown in Figure 1, in which increasing peak fronting for both acetylsalicylic acid and benzoic acid for 50, 75 and 100-µL injections of the two model analytes in

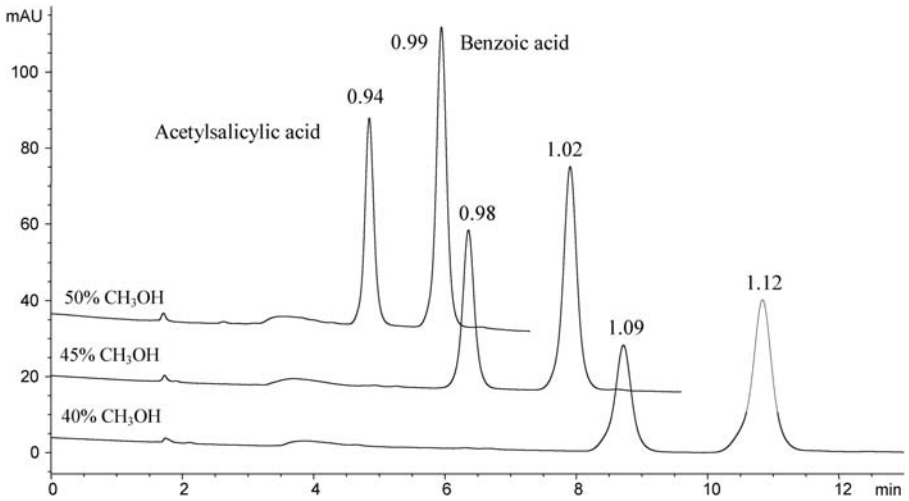

Figure 2. Chromatograms for acetylsalicylic acid and benzoic acid injected from propylbenzene at 75 μL for different mobile phase compositions in the range of 40–50% CH_3OH. Peak symmetry (calculated with Agilent Chemstation software version B.03.02) increases toward values higher than 1 (fronting peaks) when a lower CH_3OH content is used in the mobile phase for both analytes.

propylbenzene can be observed. Nevertheless, the two peaks can be integrated with reasonable accuracy, even at 100 μL. No peak fronting was observed for phenol dissolved in benzene, or for benzyl alcohol dissolved in ethylbenzene, even for a 100-μL injection volume.

Variation of the mobile phase composition was also taken into account to observe its effects on retention and other chromatographic parameters when large volumes of aromatic solvents were injected. For example, organic modifier content variation was performed with ±10% for phenol and ±5% for acetylsalicylic and benzoic acid, injecting 1–100 μL of these analytes dissolved in the four aromatic solvents. As expected, increasing the CH_3OH content in the mobile phase leads to lower retention for the three model analytes. Injection of up to 100-μL samples of the model analytes dissolved in the four aromatic solvents is still possible with the same minimum effects on peak shape and efficiency. A linear decrease in retention with the injection volume increase was observed for all mobile phase compositions, with correlation coefficients very similar to those presented in Table II ($R^2 > 0.99$). A tendency toward larger peak distortion was observed at lower CH_3OH content. This is quantified by increased peak symmetry values corresponding to the peak fronting effect (Figure 2). The same effect was observed for all four hydrophobic diluents and phenol (30–50% CH_3OH range), but only for volumes higher than or equal to 50 μL. A possible explanation is that a low methanol content in the mobile phase leads to higher retention of the analyte in the stationary phase; this leads to a more probable interference of solubilized solvent molecules in the adsorption–desorption equilibria of analyte molecules, and hence to more pronounced peak distortion.

Overall, the dependence of the ten-base logarithm of the retention factor (log k) on the content of methanol in the mobile phase ($C_{metbanol}$) for the studied analytes obeyed the known Soczewinski equation, which can be written as:

$$\log k = \alpha - \beta C_{metbanol} \quad (1)$$

(1)where the empirical parameters α and β can be calculated from the linear regression applied to the preceding dependence. For example, the dependences of three of the studied analytes for two different injection volumes of aromatic solvents are shown in Figure 3, together with the linear regressions for each dependence. All linear regressions were characterized by high correlation coefficients (>0.9900), which means that the retention process of the analytes is not influenced by the sample solvent after its adsorption onto the stationary phase. At higher than 50% CH_3OH in mobile phase, the attempts to obtain linear dependences between log k and $C_{metbanol}$ failed, owing to the solubilization of the sample solvent in the mobile phase, which influences the partition process of analytes between mobile and stationary phases. For this reason, the injection of large volumes of aromatic solvents on the phenyl-hexyl stationary phase is possible at medium or low concentrations of the organic modifier in the mobile phase. At higher contents of methanol, the adsorption of sample solvent into the stationary phase is no longer strong, and the sample solvent begins to mix with mobile phase, which influences the retention process of the injected analytes.

These experimental data can be explained by the adsorption of aromatic solvent molecules in the phenyl-hexyl phase immediately after injection, due to the π–π and hydrophobic interactions. A simplified model of these interactions between the solvent molecule (propylbenzene) and the phenyl-hexyl moiety from the stationary phase is illustrated in Figure 4. This causes the blocking of a number of the adsorption centers, which are no longer available for the analyte to interact due to the much higher affinity between the solvent molecules and the stationary phase than the affinity between the analyte and the stationary phase. The number of the blocked adsorption centers should be proportional to the injection volume (11). Analyte molecules diffuse out of the solvent plug immediately after injection and will take part to the separation process. Hence, a linear decrease in the retention factor of the model

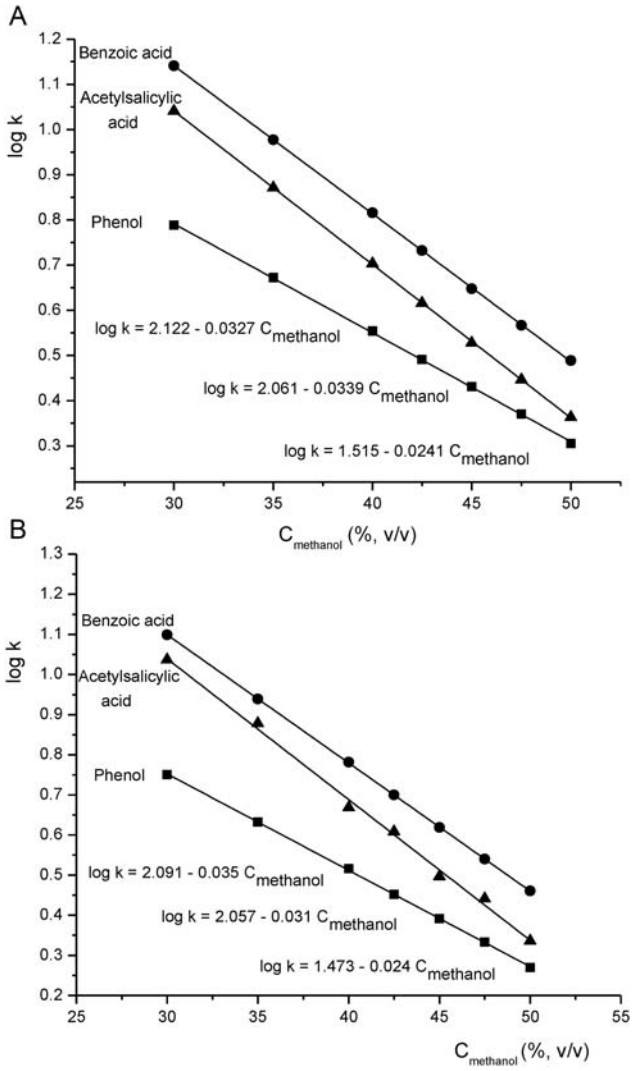

Figure 3. Linear dependences between log k on $C_{methanol}$ for: 50 μL benzene containing three analytes (A); 100 μL propylbenzene containing three analytes (acetylsalicylic acid, benzoic acid and phenol) (B).

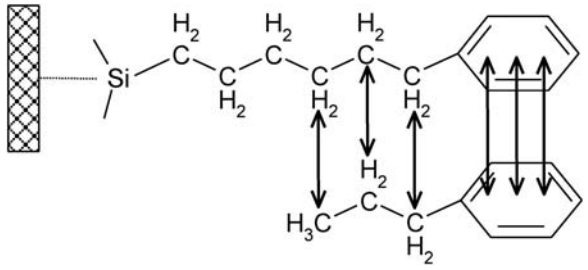

Figure 4. Hydrophobic interaction and aromatic π−π interaction between the phenyl-hexyl chain of the stationary phase and the propylbenzene molecule.

analytes takes place, proved by linear dependences between k and V_{inj}, previously discussed.

Comparing the retention factors obtained when using different diluents as sample solvent for the same model analyte, a very low decreasing tendency was observed when solvent hydrophobicity was increased in the homologous series benzene, toluene, ethylbenzene and propylbenzene; 2–3% lower k values were obtained for propylbenzene as sample diluent compared to benzene. The observed retention decrease tendency for more hydrophobic diluents can be explained by the stronger interaction between propylbenzene and phenyl-hexyl chains of the stationary phase than between benzene molecules. However, the differences are small, which can be affected by experimental errors. For this reason, the contribution of the chain–chain hydrophobic interaction between solvent and stationary phase is smaller than the π−π interaction. A comparison of the dependences of the retention factor for benzyl alcohol, for instance, on the injection volume obtained under the same chromatographic conditions on two different columns, phenyl-hexyl and octyl (C8) stationary phases, revealed the dominant role of the π−π stacking in the interaction between analyte and stationary phase, as shown in the two graphs given in Figure 5.

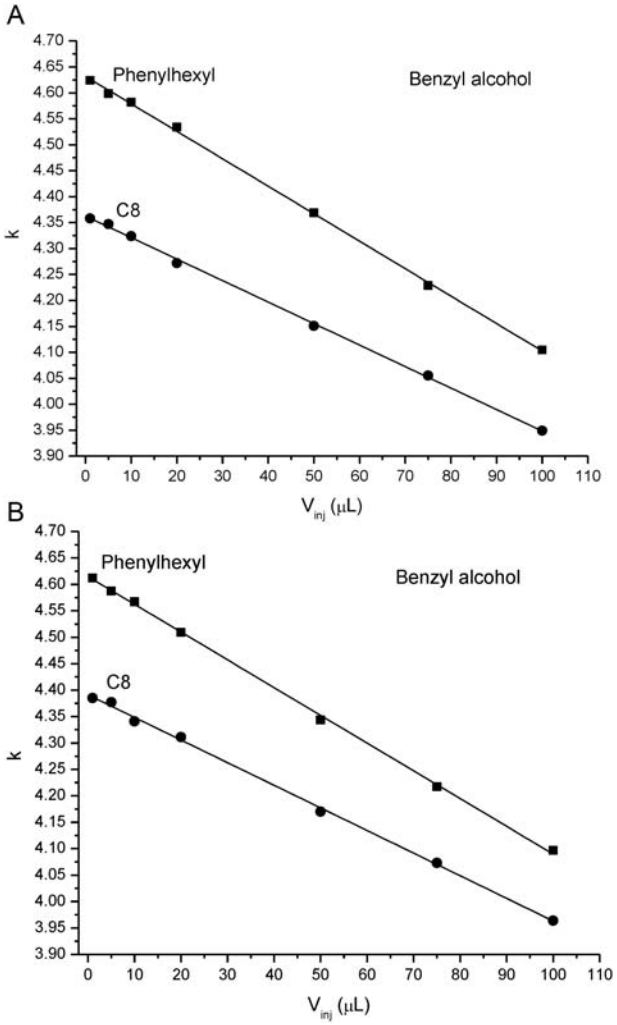

Figure 5. Comparison of the k of linear dependences on injection volume (V_{inj}) for benzyl alcohol obtained on a phenylhexyl versus C8 stationary phase with the following organic modifiers in mobile phase composition: methanol (A); acetonitrile (B).

Table III
Log K_{ow} for the Investigated Aromatic Diluents, Calculated Using ChemAxon Prediction Software (19)

Solvent	Log K_{ow}
Benzene	1.97
Toluene	2.49
Ethylbenzene	2.93
Propylbenzene	3.38

Table IV
Log K_{ow} for the Model Analytes, Calculated Using ChemAxon Prediction Software (19)

Model analyte	Log K_{ow}
Phenol	1.67
Acetylsalicylic acid	1.24
Benzoic acid	1.63
Benzyl alcohol	1.21
Codeine	1.34
Trimetazidine	0.91
4,4′-Dipyridine	1.19

Hydrophobicity (generally, expressed as ten-base logarithm of octanol–water partition constant, or log K_{ow}) of analytes participating in the retention process is also important for this approach. The values of this parameter for all seven model analytes together with that of the four aromatic solvents were computed by means of ChemAxon's prediction software, Marvin version 5.4.0.0 (Tables III and IV). Because the model analytes were chosen to be less hydrophobic than the model solvents, retention of the latter is higher in the RP-LC mechanism. This consideration was experimentally verified by measuring the retention of the four solvents in the same conditions as for the model analytes by injecting solutions of benzene, toluene, ethylbenzene and propylbenzene. The retention time values of these solvents were higher than the experimental values of the seven studied analytes.

Conclusions

Large volume injection of hydrophobic aromatic solvents like benzene, toluene, ethylbenzene or propylbenzene using phenyl-silica based stationary phases in RP-LC is possible without significant adverse effects on analye retention, efficiency or peak shape. Analyte hydrophobicity must be lower than that of the solvents; proper analyte solubility in the respective solvents is also necessary. An adsorbed solvent wash from the chromatographic column is required before a new injection is performed. The retention of all model analytes decreases proportionally with the amount of injected solvent. Good peak shapes were obtained, even at 100 μL, for most of the model analytes, with a few exceptions. Efficiency also decreases when the injection volume increases, while peak width increases slightly. Nevertheless, these adverse effects are acceptable when compared to the possibility of directly injecting samples coming from LLE dissolved in the previously mentioned solvents. The elimination of tedious sample preparation steps like solvent evaporation or residue re-dissolution leads to an increase in the accuracy of LLE procedures. Sensitivity issues can also be addressed by directly injecting large volumes of samples in LC. Increasing the solvent hydrophobicity from benzene to propylbenzene has a very small reducing effect on the retention of the model analytes, showing that the number of occupied adsorption centers is almost the same for all four aromatic solvents.

Acknowledgments

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