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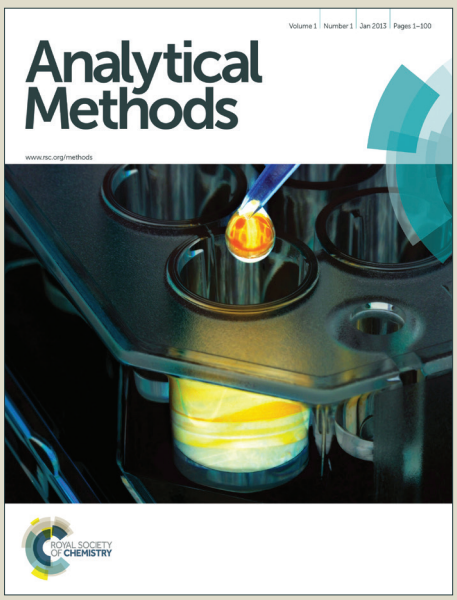

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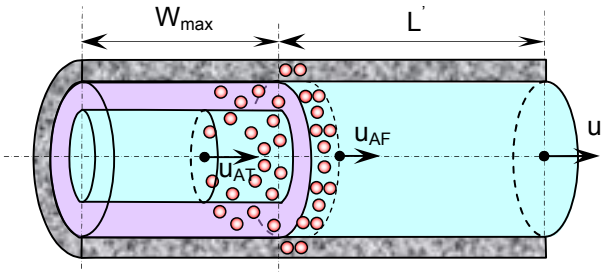

Large volume injection of immiscible diluents may be described as an on-line Reversed Phase Supported Liquid Extraction / Liquid Chromatography process. A mathematical model explaining reduction of retention with the injected volume was proposed. The model was tested on homologous *para*-alkyl hydroxybenzoates dissolved in liquid alkanes.

Revisiting large volume injection in non-miscible diluents: an on-line reversed phase supported liquid extraction / liquid chromatography scenario

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Abstract: According to some recently published studies, large volume injection (LVI) of diluents immiscible to the eluents used in reversed phase liquid chromatography (RPLC) appears to be feasible, despite the widely accepted rules governing injection related phenomena. The process was previously described and was successfully applied in practice. The present study describes a simple theoretical model explaining LVI of diluents immiscible to the mobile phase in RPLC; the model relies on the on-line coupling of reversed phase supported liquid extraction (RP-SLE) to the chromatographic separation. The compliance of the theoretical model to experimental observations was tested by using data collected for LVI of a homologous series of *para*-hydroxyalkylbenzoates (methyl, ethyl, propyl, butyl, pentyl, hexyl and octyl congeners) in liquid alkanes (hexane, heptane, iso-octane, decane and dodecane). The SP consisted of a octadecyl chemically modified silicagel eluted with a mixture of acetonitrile:water 4:6 (v/v). Although the model mainly focuses on explaining the linear reduction of the retention time with the injected volume, some aspects relating to zone spreading and thermodynamic aspects are also discussed.

1. Introduction

Large Volume Injection (LVI) in chromatography relates to the continuous challenge of increasing method sensitivity [1,2]. Injection phenomena arising in liquid chromatography (LC) were extensively studied with respect to the strength [3-7] and viscosity [8-10] of the sample diluent (D) and their recurrent impact on the chromatographic parameters illustrating retention, band spread, and peak shape. The injection produces a thermodynamic disturbance in the chromatographic system. Column loadability in a LVI complex phenomenon depends directly on its intrinsic characteristics (length, internal diameter, particle size, and phase ratio), mobile phase (MP) composition and elution conditions, D characteristics, and the specific properties of the analytes (A). LC injection phenomena should be also considered in close relationship to the widely applied sample preparation methods, primarily delivering the target compounds in a wide variety of organic solvents [11]. Moreover, removal of solvents producing unwanted phenomena in LC and their replacement by adequate ones strongly impacts on the characteristics of the sample preparation approaches and directly influences the overall accuracy and precision.

The possibility of using immiscible D to the MP in RPLC was already demonstrated [12-14]. The process was successfully applied in the pharmaceutical field [15-19] and in bioanalysis [20-24]. Injection in immiscible D was also used for estimation of the hydrophobic characteristics of a series of pharmaceutical compounds [25]. However, determination of the retention corresponding to a hypothetical condition assuming the absence of the D is more related to the intrinsic behavior of the analyte in the chromatographic column, rather than an illustration of injection related phenomena.

The possibility of introduction in the chromatographic column of large volumes of samples in immiscible D to the MP was explained through the competitional equilibria between the analyte and the D molecules for the adsorption sites in the stationary phase (SP) [12]. Consequently, the process becomes experimentally possible, without affecting peak shapes, if the affinity of the D for the SP is higher than that of the analyte. As a direct consequence, a part of the SP in the column's head becomes unavailable for the compounds contained in the sample, their retention linearly decreasing with the increase of the injection volume. It results that an essential condition for achieving LVI of immiscible D in RPLC is an increased hydrophobic character of the D compared

to analytes. The process generally occurs with a loss in the peak efficiency, although band focusing was also reported in some specific conditions [13]. In some specific cases, acceptable peak fronting or tailing may be observed.

However, these explanations seem to only superficially describe the real chromatographic phenomena. A new theoretical model of LVI in immiscible D in the RPLC MP is presented herein. This new approach assumes that immediately after the filling of the column's head with the sample dissolved in an immiscible D, the later acts as a liquid phase immobilized on the SP. The MP penetrates the D plug from behind through running channels, with the analyte(s) being extracted between the D and the MP. Once extracted from the D, the analytes are separated in the part of the chromatographic column remaining available for interaction. The scenario involves the creation, after injection, in the column's head, of a reversed phase supported liquid-liquid extraction (SLE) "cartridge," followed by the on-line removal of analytes by the MP and their separation in the remaining part of the chromatographic column. The theoretical model focuses on explaining the linear reduction of the retention in the system when the injected volume increases. To validate the theoretical model, a homologous series of *para*-hydroxyalkyl benzoates (methyl, ethyl, propyl, butyl, pentyl, hexyl and octyl congeners) dissolved in liquid alkanes (hexane, heptane, iso-octane, decane and dodecane) were used. The SP consisted of an octadecyl chemically modified silicagel eluted with a MP consisting of a mixture of acetonitrile:water 4:6 (v/v). The thermodynamics of the phenomena as well as band spreading are also discussed.

2. Experimental

2.1. Reagents

Acetonitrile (HPLC gradient grade), hexane, heptane, *i*-octane (all Lichrosolv® grade), decane (for synthesis) and dodecane (Msynth®plus) from Merck (Darmstadt, Germany) were used during experiments. Water for chromatography (resistivity of minimum 18.2 MΩ and total residual organic carbon content – TOC – of maximum 30 ng mL^{-1}) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument (TKA Instruments as part of Thermo Fischer Scientific, Niederelbert, Germany). Methyl, ethyl, propyl and butyl *para*-hydroxybenzoates (further denoted MeP, EtP, PrP, and BuP, respectively) of secondary standard pharmaceutical purity grade

were purchased from Sigma-Aldrich (Taufkirchen, Germany). The pentyl *para*-hydroxybenzoate (PeP) was obtained from Santa Cruz Biotechnology Inc. (Delaware, U.S.A.). The hexyl congener (HeP) was purchased from TCI Europe N.V. (Zwijndrecht, Belgium) while the octyl one (OcP) was obtained from Alfa Aesar (Massachusetts, U.S.A).

2.2. Equipment

Experiments were performed with an Agilent 1260 Infinity series LC/MWD (Agilent Technologies, Waldbronn, Germany) system consisting of the following modules: quaternary pump (G1311B), automated injector (ALS - G1329B), column thermostat (TCC - G1316C), and a multi-channel UV-Vis detector (DVL - G1365D). Occasionally, the refractive index detector (RID – G1362A) was used to monitor the retention behavior of the D. System control and data acquisition were made with the Agilent Chemstation for LC 3D, version 04.03(16).

2.3. Chromatographic experiments

A Zorbax SB-C18 column (50 mm L x 4.6 mm i.d. x 1.8 μm d.p.) from Agilent Technologies (cat. no. 827975-902) was used and thermostated at 25 ℃. The SP consists on spherical silica particles (1.8 μm) with a pore size of 80 Å densely covered (10% carbon load) with octadecyl moieties. The MP consisted of a mixture of acetonitrile and water, in the volumetric ratio of 4:6. The flow rate was 1.5 mL min^{-1}. Elution was made under isocratic conditions. To prepare the column for consecutive injections, the composition of the MP was brought to 100% acetonitrile in a 0.1 min stepwise ramp, followed by a period of time used to eliminate the D plug from the column. Time required for elimination of D from the column depends upon the injected volume. To accommodate experiments with working in unassisted sequences, the period for D exclusion was set to 20 min. To shorten this period, a flow rate stepwise increase simultaneous to the jump at 100% acetonitrile may be used. Next, the composition of the MP was set at the initial mixing ratio (4:6) followed by 3 min of equilibration. Spectrometric UV detection was applied, using 270 nm as analytical wavelength. For samples dedicated to 1 μL injection volume, stock solutions of the analyte's mixture of 2 mg mL^{-1} each in methanol (for hexane, heptane and iso-octane) and in ethyl acetate (for decane and dodecane) were 1/20 diluted with the respective D. From these intermediate stock solutions, 1/5, 1/10, 1/20, 1/50 and 1/100 dilutions in the appropriate D were made, in order to keep constant the absolute amounts of analytes when 5, 10, 20, 50 and 100 μL injection volumes

were applied. Separately, serial injection of 1, 5, 10, 20, 50, and 100 μL mixtures of the analytes in the MP was also achieved (individual injected amounts were kept constant during experiments).

2.4. Complementary approaches

The distribution constants (K) of the considered compounds between heptane and the acetonitrile/water mixture 4:6 (v/v) were determined according to the shake flask method after vortexing during 24 hrs (vortexing speed 500 rpm) of equal volumes of phases (the initial concentration of each compound in the organic layer was 10 μg mL^{-1}). Quantitative determinations of the compounds were made in both layers separately, by means of the chromatographic method. The mass balance for the sum of the determined compounds in the two phases with respect to the initial amounts varied from 98.1 to 100.6% in recovery.

Retention of model compounds was studied separately when the MP was deliberately saturated with heptane. Saturation was achieved through vortexing the pre-mixed MP (acetonitrile/water = 4/6, v/v) with heptane during 24 hrs, in a volumetric ratio of 17:1. The injected volume was 1 μL from a solution in methanol at 1000 μg mL^{-1} from each compound of the homologous series. Subsequent injections were made in order to obtain constant retention times.

For the thermodynamic studies, BuP was used dissolved in heptane, and injected in incremental volumes of 20, 40, 60, 80 and 100 μL. The temperature interval used in the study was between 20 to 45 °C, in 5 °C increments. Van't Hoff plots were used for calculation of apparent standard enthalpies and entropies.

3. Results and discussions

3.1. Rationale for the choice of the experimental conditions

Para-hydroxyalkyl benzoates homologous series were used as model compounds, covering a large scale of hydrophobicity. The values of the logarithms of their partition coefficients between *n*-octanol and water (log K_{ow}), calculated according to the fragment theory (KOWWIN v1.67, Environmental Protection Agency, U.S.A) are, in order: MeP - 2.00; EtP - 2.48; PrP - 2.98; BuP - 3.47; PeP - 3.96; HeP - 4.45; OcP - 5.43.

The chosen D are characterized by the following computed log K_{ow} values: hexane - 3.29;

heptane - 3.78; iso-octane - 4.09; decane - 5.25; dodecane - 6.23. According to the previous theory, no suitable results should be obtained in cases where the log K_{ow} of the analyte is higher than the log K_{ow} of the D.

The column was chosen with a minimal length (50 mm) for two main reasons. First of all, it was chosen to produce lower retention and to achieve separation of the model compounds under isocratic conditions while keeping the MP composition with a highest percentage of water. This has the effect of producing the lowest solubility of the D in the MP. Secondly, the column geometry makes injections of 10 to 100 μL possible, representing a significant part of the kinetic void volume. The column void volume (V_0) was 447 μL, which was determined by injection of a solution of potassium nitrate. As such, the D injected volumes represented 2.24 to 22.4% from the void volume.

3.2. The theoretical model

The theoretical model being advanced herein is based on the scenario of an RP-SLE process on-line coupled to RPLC (see Figure 1).

The steps of the process are: (I) the transport of the D plug in the head of the chromatographic column (filling - Figure 1 A and B); (IIa) inflation of the D plug produced by the penetration of the MP (formation of channels) and its diffusion in the SP; (IIb) the liquid-liquid extraction (LLE) of the analyte from the D in the MP until the D/MP front interface is reached (stages IIa and IIb are simultaneous) – see Figure 1C; (IIIa) beginning of reinjection of the MP containing the extracted analyte into the rest of chromatographic column and continuation of the LLE process; (IIIb) LLE process ends, the normal chromatographic process occurs in the remaining portion of the column.

Figure 1

Some general simplifying assumptions are necessary: *i)* the D completely replaces the MP during loading (no intra and extra particle entrapping of the MP arises); *ii)* the D plug remains immobile after its transport in the column's head and its inflation (due to its larger affinity to SP as compared with that of MP); *iii)* the reciprocal solubility of the D and the MP should be considered

as negligible; *iv)* the hydrophobic character of the D is significantly similar to the SP character; *v)* the model ignores the effect of the longitudinal/axial and radial/transversal mass transfer widening the analyte zone; thus, the short range (at the order of magnitude of particle size dimensions) mass transfer via radial/transversal diffusion is considered instantaneous but axial/longitudinal diffusion is considered 0; *vi)* the number of the MP penetrating channels through the D soaking the SP is significantly similar to the number of chromatographic elution channels through the packing material; *vii)* the SP contribution to the analyte partition in the D plug is negligible (the surface retention activity of the SP is quenched by the presence of the D existing in a much larger amount); the contribution of the SP to the dilution effect of the analyte in the D zone is negligible; *viii)* no fingering effects at the interfaces between the immiscible liquid zones were considered.

The elimination of the fingering effects from the theoretical model was not made for simplification reasons, only. Fingering effects which may appear at the front interface between the D and the MP are hindered by the consecutive inflation of the D plug (step IIa). Fingering effects arising at the rear interface between the MP and the D are hindered by the penetration of the MP in the D plug and the channel formation process. Experimental results confirm the absence of the fingering effects.

The following mathematical relationships were used to describe the RP-SLE process on-line coupled to RPLC.

During the first stage (I) of the process, the following relationships are established:

The D maximum longitudinal spread on loading ($W_{\max}^{D,I}$) is $W_{\max}^{D,I} = \frac{V_{inj}}{V_0} L = \alpha L$ (1)

where V_{inj} is the injected volume, V_0 is the void column volume, L is the column length and α is further referred as reduced injected volume.

The D maximum filling time in the column's head ($t_{\max}^{D,I}$) is $t_{\max}^{D,I} = \frac{V_{inj}}{F} = \frac{V_{inj}}{V_0}\frac{V_0}{F} = \alpha t_0$ (2)

where F is the MP flow rate and t_0 is the column void time.

Obviously, the filling speed of the D plug is the MP speed ($u = \frac{F}{S_0} = \frac{W_{\max}^{D,I}}{t_{\max}^{D,I}} = \frac{L}{t_0}$), where S_0 is the effective MP cross-section through the column packing.

The MP evolution during stage (IIa) (channel formation) of the process may be described by the following relationships:

$$\Delta u = u_1 - u = \frac{F}{S_1} - \frac{F}{S_0} = \frac{F}{\gamma_1 S_0} - \frac{F}{S_0} = u\frac{1-\gamma_1}{\gamma_1} \quad (3)$$

where u_1 is the speed of the MP front during penetration of the D plug and channel formation, S_1 is the effective MP cross-section after D plug penetration and $\gamma 1$ is the reduced cross-section S_1/S_0.

The period needed for MP channels formation should thus be considered as:

$$t_{\max}^{D,IIa} = \frac{W_{\max}^{D,I}}{\Delta u} = \frac{\alpha L}{u}\frac{\gamma_1}{1-\gamma_1} = \alpha t_0 \gamma_1 \iota, \quad \text{where } \iota = \frac{1}{1-\gamma_1} = \frac{S_0}{S_0 - S_1} = \frac{S_0}{S_2} = \frac{1}{\gamma_2} \text{ is the inflation factor} \tag{4}$$

The cumulative period needed for D filling and MP channel formation through the D plug is:

$$t_{\max}^{D,I+IIa} = \alpha t_0 + \alpha \gamma_1 \iota t_0 = \alpha t_0 (1 + \gamma_1 \iota) = \alpha t_0 \iota \tag{5}$$

The longitudinal spatial spread of D during steps (I) and (IIa) may be calculated as

$$W_{\max}^{D,I+IIa} = \alpha L + u t_{\max}^{D,IIa} = \alpha L + \alpha t_0 \gamma_1 \iota \frac{L}{t_0} = \alpha L (1 + \gamma_1 \iota) = \alpha L \iota \tag{6}$$

During the liquid-liquid extraction step (IIb), the evolution of the analyte's (A) rear front may be described starting from the mass conservation relationship:

$$S_1 [A]_{MP} (u_1 - u_{AT}) = S_2 [A]_D u_{AT} \tag{7}$$

where S_1 is the effective MP cross-section after D plug penetration, S_2 is the immobilized D cross-section after MP penetration through the plug, u_{AT} is A rear front speed in MP until extraction from D layer ends, and $[A]_{MP}$ and $[A]_D$ are equilibrium concentrations of A in MP and D, respectively.

Consequently

$$\frac{u_1 - u_{AT}}{u_{AT}} = \frac{S_0 - S_1}{S_1} K = \frac{1-\gamma_1}{\gamma_1} K, \quad \text{as } u_1 = \frac{u}{\gamma_1} \text{ it results that } u_{AT} = \frac{u}{\gamma_1 + K(1-\gamma_1)} \tag{8}$$

where $K = [A]_D/[A]_{MP}$ is the liquid-liquid partition constant of A between MP and D.

Thus, the compression speed of the analyte front is $\Delta u = u_{AT} - u = \frac{[1 - \gamma_1 - K(1-\gamma_1)]u}{\gamma_1 + K(1-\gamma_1)}$ (9)

The maximum rear analyte depleted zone width $W_{A,void}^{IIb}$ may be calculated as:

$$W_{A,void}^{IIb} = u_{AT} t_{\max}^{D,IIa} = \frac{u}{\gamma_1 + K(1-\gamma_1)} \alpha t_0 \gamma_1 \iota = \frac{\alpha L \gamma_1}{[\gamma_1 + K(1-\gamma_1)]} \frac{1}{(1-\gamma_1)} \tag{10}$$

The compressed analyte zone is characterized by the width W_A^{IIb}, resulting from the following relationship:

$$W_A^{IIb} = W_{\max}^{D,I+IIa} - W_{A,void}^{IIb} = \alpha L \iota - \frac{\alpha L \gamma_1}{\gamma_1 + K(1-\gamma_1)} \frac{1}{(1-\gamma_1)} = \frac{\alpha L K}{\gamma_1 + K(1-\gamma_1)} \tag{11}$$

For the reinjection stage (IIIa), the mass conservation relationship gives:

$$(u - u_{AF})[A]_{MP} S_0 = u_{AF} [A]_{SP} S, \quad \text{from which } \frac{u - u_{AF}}{u_{AF}} = K_0 \gamma \tag{12}$$

where u_{AF} is the analyte front speed after reinjection, S is the SP cross-section, $[A]_{MP}$ and $[A]_{SP}$ are equilibrium concentrations of A in MP and SP, respectively, while $K_0 = [A]_{SP}/[A]_{MP} = k\gamma^{-1}$; $\gamma = V_{SP}/V_{MP}$, is the chromatographic equilibrium constant of A between SP and MP. Thus, u_{AF} may be written as:

$$u_{AF} = \frac{u}{1+K_0\gamma} = \frac{u}{1+k} \tag{13}$$

The virtual duration of the reinjection process of the analyte (t_A^{IIIa}) is:

$$t_A^{IIIa} = \frac{W_A^{IIb}}{u_{AT}} = \frac{\alpha LK}{\gamma_1 + K(1-\gamma_1)} \frac{\gamma_1 + K(1-\gamma_1)}{u} = \alpha K t_0 \tag{14}$$

The maximum spatial width of the analyte's zone after reinjection W_A^{IIIb} is:

$$W_A^{IIIb} = u_{AF} t_A^{IIIa} = \frac{u}{1+K_0\gamma} \alpha K t_0 = \frac{\alpha KL}{1+k} \tag{15}$$

The remaining length of the column available for the chromatographic separation of the analyte L^{IIIb} is:

$$L^{IIIb} = L - W_{\max}^{D,I+IIa} = L - \alpha L\iota = L(1-\alpha\iota) \tag{16}$$

The retention time corresponding to the remaining length of the column available for the chromatographic separation is $t_R^{IIIb} = t_R(1-\alpha\iota) = (1-\alpha\iota)(1+k)t_0$ (17)

It follows that the apparent retention time of the analyte t_R^{app} may be computed according to the following relationship:

$$t_R^{app} = t_{\max}^{D,I+IIa} + \frac{t_A^{IIIa}}{2} + t_R^{IIIb} = \alpha t_0 \iota + \frac{\alpha K t_0}{2} + t_R(1-\alpha\iota) \tag{18}$$

The $\frac{\alpha K t_0}{2}$ term is actually the half width (in time units) of the peak, if diffusion does not contribute to zone spreading as the model considers. If considering $t_R^{app} = t_0(1+k_{app})$, it follows that:

$$k_{app} = \frac{\alpha K}{2} + k(1-\alpha\iota) = \alpha\left(\frac{K}{2} - \iota k\right) + k \tag{19}$$

If the linear relationship [19] must fit the experimental findings, the slope $\frac{K}{2} - \iota k$ should be negative (the apparent retention factor decreases linearly with the increase of the injected volume). This is equivalent to the situation where $K < 2\frac{S}{S_2}K_0$ (20)

Experimental results show that the apparent retention factor, k_{app}, is less influenced by the distribution constant K, its functional dependence on α being mainly due to the ιk term (the inflation factor and the chromatographic retention factor). As a direct consequence, one can conclude that

the variation of the apparent retention with α ($\frac{dk_{app}}{d\alpha}$) is mainly determined by the inflation factor (ι). The inflation factor is a geometrical parameter which does not depend directly on any chromatographically related parameter, but only on the shear forces developed at the D/MP interface (influenced by viscosity) and the interfacial forces of the D/MP interface. A reasonable explanation of the stabilization to a given thickness of the D film covering the SP can be the so-called "disjoining pressure".

3.3. Correlation with experimental results

As illustrated in Table 1, linear relationships between k_{app} and α are established for all D and studied compounds. This is not surprising at all, as the linear decrease of the retention factor with the injected volume was observed during the earlier works relating to the use of immiscible D in RPLC [12,13,19,25]. One can observe that the coefficients describing the correlations between experimental data are higher than 0.99.

Table 1

The partition constants (K) of the analytes between heptane and the MP were available from the shake flask experiments. The chromatographic equilibrium constants (calculated with $K_0=k\ \gamma^{-1}$ i.e. using the formalism of the partition based retention) were also available from the injection of the analytes dissolved in the MP. The retention factor, k, and the phase ratio (γ) are calculated using a void volume of 0.447 mL, and a SP volume of 0.1 mL (an approximation of the bulk octadecyl moieties volume covering the surface of the silica material). It is thus possible to compute some of the key parameters of the model, more precisely ι, γ_2, $W_{\max}^{D,I+II}$. Results are illustrated in Table 2.

Table 2

It can be observed that conditions described in eq. 20 are fulfilled in all cases. The chromatographic equilibrium constants (K_0) are significantly higher than the LLE ones (K). As one

can observe from the γ_2 calculations, the cross-section of the column occupied by the supported D (S_2) is roughly one-half of the column cross-section occupied by the MP (S_0).

ι (and its inverse γ_2) values should depend mainly on the nature of the D and the nature of the SP. Experimental results in Table 2 support this assumption, with ι and γ_2 values being significantly similar for all analytes at injected volumes equal or higher than 10 μL. The relative standard deviations (RSD%) computed for the ι and γ_2 data sets are placed below 15% (n=28). Data obtained for 1 and 5 μL injection volumes for all analytes were deliberately discarded. Such volumes are most probably not homogeneously occupying the cross-section of the chromatographic column during the transfer of the D plug in the head of the column, immediately after injection. Such a process should be the basis, at least for the last eluting compounds (for which the LLE from the D plug to the MP remains unfavorable), of severe peak shape distortion, and fits to the experimental observations (Figure 2 illustrates peak shapes corresponding to the last two eluting compounds when injecting 1 μL from all used D).

Figure 2

Injection volumes higher than 10 μL in all tested D lead to fair peak symmetry and efficiency for the considered compounds (see Figure 3 and ***Electronic Supplementary Information, Part 1***). It appears that the condition for successful chromatographic results stated in previous works ($\log K_{ow}^{Diluent} > \log K_{ow}^{Analyte}$) does not necessarily apply.

Figure 3

From Table 2 one can see that the inflated zone of the D ($W_{\max}^{D,I+II}$) for the 100 μL injected volume is occupying almost one-half of the chromatographic column. Reduction of the column length produces a proportional decrease of the peak efficiency. When injecting large volumes of solutions in the MP, the first four eluting compounds suffer a reduction of peak efficiency. This is due to the inability of the SP to re-focus analytes on injection (Table 2 and ***Electronic***

Supplementary Information, Part 2). For the earlier eluting compounds (poorly retained in D and SP), on injection of immiscible D, the MP channels formed in the D plug are focusing analytes (eq. 9), due to their favorable partition from the D to the MP. Consequently, the apparent peak efficiency (N_{app}) will increase with the increase of the injected volume, compared to the situation when the MP is used as D. For the later eluting compounds (strongly retained in D and SP), partition from the D to the MP during the channels' formation is not favored, but the SP in the head of the remaining chromatographic column focuses the analytes and compensates (at least partially) for the band spread. Consequently, the initial spread of the analyte zone due to filling of a large amount of D may not be entirely compensated by either compression or refocusing, and the virtual re-injected volume can be larger than V_{inj} if $K>>1$.

The simplifying conditions assumed by the mathematical model (specifically, the assumptions relying on the reciprocal lack of solubility between the D and the MP, on one hand, and the immobility of the D plug after column percolation and inflation process, on the other hand) are not obeyed in experimental practice. As illustrated in ***Electronic Supplementary Information, Part 3***, around 25 min after injection of 100 μL of heptane, the D plug starts to be evacuated from the column. This can be observed under RID conditions, but also in the UV trace (as a noisy baseline due to formation of a micro-emulsion of the D in the MP). In real conditions, the D plug should act as a MP saturator. A MP saturated with the D would also decrease the chromatographic retention. As a result it was decided to study the effect of the saturation of the MP with the D, under conditions of small volume injection in methanol (1 μL). Experimental conditions were described in *Complementary approaches* under the *Materials and methods* section. Results are presented in the ***Electronic Supplementary Information, Part 4***. As expected, on repetitive injections, retention continuously decreases until equilibrium of the partition of the saturating D between phases is obtained. Surprisingly, the equilibration period is extremely long, largely exceeding 800 void volumes. Under such conditions, for the assumed on-line RP-SLE/RPLC scenario, MP saturation with the D should not produce any measurable effects and fully explains why the experimental results fit to the proposed mathematical model. From the number of void volumes needed to equilibrate the column when the MP is saturated with D one can also conclude that the displacement mechanism of the D plug is mainly based on mechanically driven forces and not

through a chromatographic process.

3.4. Thermodynamic considerations

To have better insight, a thermodynamic study was also conducted. The global thermodynamic behavior of the system should take into account the individual contributions of both consecutive processes, the LLE extraction and the chromatographic separation on the remaining available chromatographic column. A study of the relationships between the logarithms of the apparent retention factor (ln k_{app}) versus the inverse of the temperature was carried out (van't Hoff plots). The considered temperature interval ranged from 20 to 45 °C (in steps of 5 °C). The chosen model compound was BuP dissolved in heptane and injected in volumes of 20, 40, 60, 80 and 100 μL, respectively. A comparison with the situation involving only the chromatographic separation, meaning the injection of 1 μL of a solution of the model compound in methanol, is also presented. Resulting data are given in Table 3. The graphical plots are also provided in ***Electronic Supplementary Information, Part 5***.

Table 3

Surprisingly, all the van't Hoff plots were linear, despite the two different processes acting additively (LLE and the chromatographic separation). The resulting standard enthalpies (ΔH^0) vary from -11.52 to -13.32 kJ, with increasing the injected volumes from 20 to 100 μL. The entropic terms were determined through the relationship $\Delta S^0 = R\left(A - \ln\frac{1}{\gamma}\right)$ considering the volumes of the MP and SP as 0.447 and 0.1 mL, respectively, and the phase ratio being invariant with respect to the temperature. One can observe from Table 3, that the standard entropy ΔS^0 varies from -8.90 to -20.17JK^{-1} on increasing the injected volumes from 20 to 100 μL. As illustrated in Figure 4, the relationship between ΔH^0 and ΔS^0 was found to be linear (correlation coefficient of 0.9974) when considering injection volumes in heptane ranging from 20 to 100 μL. Such a behavior may be explained through the enthalpy-entropy compensation effects [26]. Consideration of data resulting after injections of 1 μL of solutions in methanol (the empty circle in Figure 4) negatively affects the linearity of the functional relationship (correlation coefficient is reduced to 0.9892).

Figure 4

The plot of the natural logarithm of capacity factors of the solute (BuP) measured at a given temperature under different injection volume conditions against the corresponding enthalpy change should appear linear when compensation occurs. It has been stated [26] that, for purposes of enhancing the accuracy of the experimental data, the working temperature should be placed near the harmonic means of the temperature values used in the van't Hoff study. As illustrated in the ***Electronic Supplementary Information, part 6***, the plot of the logarithm of the apparent retention factor ($\ln k_{app}$) at 30 °C (the computed harmonic mean was 32.26 °C) versus ΔH^0 is described by a linear relationship characterized by a correlation coefficient of 0.9884, in the case of increasing injection volumes of heptane. If the point corresponding to injection of 1 μL solution in methanol (accounted only for the chromatographic separation process) is added to the plot, the correlation coefficient is reduced to 0.9609.

One can conclude that the thermodynamic study revealed the existence of the LLE and the RP chromatographic distinctive stages. As both stages are based on similar interactions of the analyte distributed between a hydrophobic phase (D and SP) and the MP, van't Hoff plots remain linear. The slight increase of the enthalpy on increasing the D volume is well compensated by the entropy change. Through analyzing the compensation plots, the small difference between the chromatographic separation taken alone and the on-line coupling between RP-SLE and RPLC resulted in a reduction of the correlation coefficients. A large entropy change indicates that the solute molecules are retained with less random movement in the SP than they were in the D layer.

3.5. Hydrophobicity indicating scale

From equation (19) it results that the slope of the linear regression $k_{app}=f(\alpha)$ depends on the retention factor of the model compound in the chromatographic column and its partition constant from the MP to the D (K). Consequently, the slope of the linear regression should globally express the affinity of the analyte towards hydrophobic media (the D and the SP), or towards the hydrophilic MP. Thus, a correlation between the log K_{ow} of the analytes and the slope of the linear regression $k_{app}=f(\alpha)$ should be observable. Figure 5 illustrates the correlation between the calculated log K_{ow} values of the target compounds and the logarithms of the slopes of the linear

regressions $k_{app}=f(\alpha)$, taken as absolute values, when using heptane as D on injection. One can observe the excellent correlation between the two sets of data.

Figure 5

Similar correlations were established between log K_{ow} and the logarithms of the absolute slopes resulting from injection of all analytes in the other D (see ***Electronic Supplementary Information, Part 7***). In fact, the data generated in all D are similar, as long as their hydrophobic characteristics are similar with respect to the hydrophobic characteristics of the SP. It appears that the slope of the linear relationship between k_{app} and α may successfully act as a hydrophobicity descriptor.

4. Conclusions

Large volume injection (LVI) of immiscible D in reversed phase liquid chromatography (RPLC) may be successfully described by means of a reversed phase supported liquid extraction process (RP-SLE) on-line coupled to the chromatographic separation. The simple mathematic model being developed fully explains the linear relationship experimentally established between the apparent retention factor (k_{app}) and the reduced injected volume ($\alpha=V_{inj}V_0^{-1}$). The model is based on the immobilization of the D plug immediately after its transfer in the column's head, followed by the formation of MP channels. The formation of MP channels leads to inflation of the D plug and allows liquid-liquid extraction of the analytes. The chromatographic separation follows the SLE process in the part of the column remaining after inflation of the D plug up to its maximal extent. The model allows calculation of the column cross-section occupied by the D. The model was verified on a homologous series of *para*-alkyl hydroxybenzoates, using hexane, heptane, *iso*-octane, decane, and dodecane as D. A rapid liquid-liquid extraction from the D in the MP leads to analyte focusing and improves band spreading in the case of rapidly eluting compounds, as compared to the situation of injection of large volumes of samples dissolved in the MP. For analytes poorly extracted from the D by the MP, band spreading is limited by re-focusing in the SP. It was experimentally proven that D saturation of the MP is not responsible for the retention factor

reduction in the given conditions. The SLE and RPLC processes consecutively acted according to the proposed mechanism, and are observable in the enthalpy-entropy compensation plots, if compared to the chromatographic separation conditions taken alone. It also results that the logarithm of the slope of the linear relationship between k_{app} and α, taken as absolute value, may successfully act as a hydrophobicity descriptor. LVI of immiscible D in RPLC is also one of the most direct proofs that the chromatographic retention mechanism is based on adsorption. In the experimental conditions, the adsorption based chromatographic retention is "quenched" by the D.

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Table 1. Characteristics of the linear regressions established between the apparent retention factor (k_{app}) and α (reduced injected volume as the ratio between injected volume - V_{inj} and the column void volume – V_0, $\alpha=V_{inj}V_0^{-1}$).

Diluent	Characteristics of the linear regression $k_{app}=f(\alpha)$	Model compounds						
		MeP	EtP	PrP	BuP	PeP	HeP	OcP
Hexane	Slope	-2.387	-5.144	-10.912	-22.374	-45.845	-95.813	-392.974
	Intercept	1.26	2.33	4.57	9.06	18.00	35.88	131.34
	Correlation Coefficient	0.9976	0.9990	0.9995	0.9997	0.9997	0.9996	0.9991
Heptane	Slope	-2.295	-5.045	-10.715	-21.925	-44.822	-93.271	-411.107
	Intercept	1.27	2.35	4.60	9.09	18.09	35.96	140.45
	Correlation Coefficient	0.9964	0.9984	0.9994	0.9996	0.9995	0.9996	0.9997
Iso-Octane	Slope	-2.114	-4.796	-10.366	-21.387	-43.902	-92.047	-409.310
	Intercept	1.26	2.34	4.59	9.10	18.08	36.08	142.22
	Correlation Coefficient	0.9960	0.9987	0.9996	0.9999	0.9998	0.9998	0.9997
Decane	Slope	-2.025	-4.970	-11.103	-23.005	-47.240	-99.732	-444.952
	Intercept	1.51	2.72	5.23	10.26	20.32	40.54	160.11
	Correlation Coefficient	0.9938	0.9984	0.9996	0.9999	0.9998	0.9997	0.9996
Dodecane	Slope	-2.137	-5.102	-10.971	-22.113	-46.101	-97.501	-445.343
	Intercept	1.528	2.735	5.250	10.297	20.402	40.694	161.327
	Correlation Coefficient	0.9979	0.9997	0.9996	0.9998	0.9999	0.9999	0.9997

Table 2. Different process values determined for the model compounds as resulting from the serial increase of the injection volumes in heptane (as diluent) and mobile phase (abbreviations are given in the text).

	V_{inj} (μL)	α x 10^3	k	k_{app}	K	K_0	K_0/K	ι	γ_2	Mean ι	Mean γ_2	$W_{max}^{D,I+II}$ (αLι)	L^{IIIb} (mm)	L^{IIIb}/L	N_{app}	N
MeP	1	2.237	1.27	1.27	0.011	28.5	2537.6	-	-	M=2.0; s=0.13; RSD%=6.7	M=0.508; s=0.04; RSD%=7.0	0.27	49.73	0.995	7128	6988
	5	11.186		1.25				-	-			1.35	48.65	0.973	5757	5602
	10	22.371		1.21				2.1	0.470			2.70	47.30	0.946	5550	5087
	20	44.743		1.16				2.0	0.513			5.39	44.61	0.892	5096	3860
	50	111.857		0.98				2.1	0.486			13.48	36.52	0.730	4830	1541
	100	223.714		0.77				1.8	0.564			26.96	23.04	0.461	4577	485
EtP	1	2.237	2.34	2.35	0.028	52.4	1890.9	-	-	M=2.2; s=0.09; RSD%=4.2	M=0.465; s=0.02; RSD%=4.0	0.27	49.73	0.995	8845	7951
	5	11.186		2.31				-	-			1.35	48.65	0.973	7569	7030
	10	22.371		2.23				2.1	0.472			2.70	47.30	0.946	6944	6800
	20	44.743		2.12				2.1	0.479			5.39	44.61	0.892	6972	5465
	50	111.857		1.74				2.3	0.433			13.48	36.52	0.730	6113	2671
	100	223.714		1.24				2.1	0.475			26.96	23.04	0.461	4817	906
PrP	1	2.237	4.57	4.59	0.062	102.2	1661.3	-	-	M=2.3; s=0.11; RSD%=4.8	M=0.443; s=0.02; RSD%=4.8	0.27	49.73	0.995	9601	8711
	5	11.186		4.50				-	-			1.35	48.65	0.973	8923	8037
	10	22.371		4.35				2.1	0.472			2.70	47.30	0.946	8576	7785
	20	44.743		4.12				2.2	0.452			5.39	44.61	0.892	7031	7003
	50	111.857		3.34				2.4	0.415			13.48	36.52	0.730	6889	4514
	100	223.714		2.23				2.3	0.435			26.96	23.04	0.461	4615	2071
BuP	1	2.237	9.04	9.06	0.144	202.3	1407.6	-	-	M=2.0; s=0.04; RSD%=1.9	M=0.419; s=0.01; RSD%=1.9	0.27	49.73	0.995	9705	8596
	5	11.186		8.92				-	-			1.35	48.65	0.973	9435	8181
	10	22.371		8.57				2.3	0.432			2.70	47.30	0.946	8581	8050
	20	44.743		8.06				2.4	0.413			5.39	44.61	0.892	7351	7744
	50	111.857		6.59				2.4	0.412			13.48	36.52	0.730	6717	6627
	100	223.714		4.22				2.4	0.418			26.96	23.04	0.461	3832	4289
PeP	1	2.237	17.95	17.94	0.318	401.6	1262.5	-	-	M=2.4; s=0.11; RSD%=4.4	M=0.417;s=0.02;RSD%=4.7	0.27	49.73	0.995	8790	8028
	5	11.186		17.81				-	-			1.35	48.65	0.973	8468	7807
	10	22.371		17.06				2.2	0.451			2.70	47.30	0.946	8001	7824
	20	44.743		15.98				2.5	0.405			5.39	44.61	0.892	6593	7669
	50	111.857		13.01				2.5	0.405			13.48	36.52	0.730	5945	7379
	100	223.714		8.11				2.5	0.407			26.96	23.04	0.461	3327	6292
HeP	1	2.237	35.81	35.69	0.690	801.1	1161.2	-	-	M=2.6; s=0.04; RSD%=1.5	M=0.386; s=0.01; RSD%=1.6	0.27	49.73	0.995	7525	7042
	5	11.186		35.31				-	-			1.35	48.65	0.973	7625	7206
	10	22.371		33.79				2.5	0.395			2.70	47.30	0.946	6542	7066
	20	44.743		31.61				2.6	0.380			5.39	44.61	0.892	5385	7077
	50	111.857		25.32				2.6	0.380			13.48	36.52	0.730	5039	6939
	100	223.714		15.22				2.6	0.388			26.96	23.04	0.461	2674	6609
OcP	1	2.237	141.34	139.80	2.938	3161.9	1076.3	-	-	M=3.1; S=0.12; RSD%=3.8	M=0.324; s=0.01; RSD%=3.8	0.27	49.73	0.995	4353	5001
	5	11.186		136.89				-	-			1.35	48.65	0.973	4542	5115
	10	22.371		131.38				3.2	0.316			2.70	47.30	0.946	4326	5015
	20	44.743		120.98				3.2	0.310			5.39	44.61	0.892	3647	4972
	50	111.857		93.47				3.0	0.329			13.48	36.52	0.730	3458	5044
	100	223.714		49.12				2.9	0.342			26.96	23.04	0.461	1413	5212
								Mean		2.4	0.423					
								s		0.33	0.05					
								RSD%		13.8	12.8					

Table 3. Data resulting from the van't Hoff representation (the logarithm of the apparent retention versus the inverse of the temperature ln k_{app} = f(1/T)) for *para*-hydroxybutyl benzoate (BuP) on injection of different volumes in heptane or methanol.

V_{inj} (μL)	Diluent	ln k	T (°C)	1/T (K^{-1})	B*	A**	r_{xy}***	ΔH^0 (kJ)	ΔS^0 (JK^{-1})	ΔG^0
1	MeOH	2.28	20	0.003411	1391.98	-2.4680	0.9972	-11.57	-8.07	-9.21
		2.20	25	0.003354						-9.17
		2.13	30	0.003299						-9.13
		2.05	35	0.003245						-9.09
		1.96	40	0.003193						-9.05
		1.92	45	0.003143						-9.01
20	Heptane	2.15	20	0.003411	1385.08	-2.5681	0.9990	-11.52	-8.90	-8.91
		2.08	25	0.003354						-8.86
		2.01	30	0.003299						-8.82
		1.93	35	0.003245						-8.77
		1.85	40	0.003193						-8.73
		1.78	45	0.003143						-8.68
40	Heptane	2.02	20	0.003411	1407.81	-2.7765	0.9994	-11.71	-10.64	-8.59
		1.95	25	0.003354						-8.53
		1.87	30	0.003299						-8.48
		1.79	35	0.003245						-8.43
		1.72	40	0.003193						-8.37
		1.64	45	0.003143						-8.32
60	Heptane	1.89	20	0.003411	1443.71	-3.0312	0.9998	-12.00	-12.75	-8.27
		1.81	25	0.003354						-8.20
		1.73	30	0.003299						-8.14
		1.66	35	0.003245						-8.07
		1.58	40	0.003193						-8.01
		1.50	45	0.003143						-7.95
80	Heptane	1.73	20	0.003411	1522.24	-3.4613	0.9999	-12.66	-16.33	-7.87
		1.64	25	0.003354						-9.17
		1.56	30	0.003299						-9.13
		1.48	35	0.003245						-9.09
		1.40	40	0.003193						-9.05
		1.32	45	0.003143						-9.01
100	Heptane	1.54	20	0.003411	1601.70	-3.9236	0.9936	-13.32	-20.17	-7.40
		1.44	25	0.003354						-7.30
		1.34	30	0.003299						-7.20
		1.31	35	0.003245						-7.10
		1.18	40	0.003193						-7.00
		1.11	45	0.003143						-6.90

* Slope of the linear relationship;

** Intercept of the linear relationship;

*** Correlation coefficient of the linear relationship.

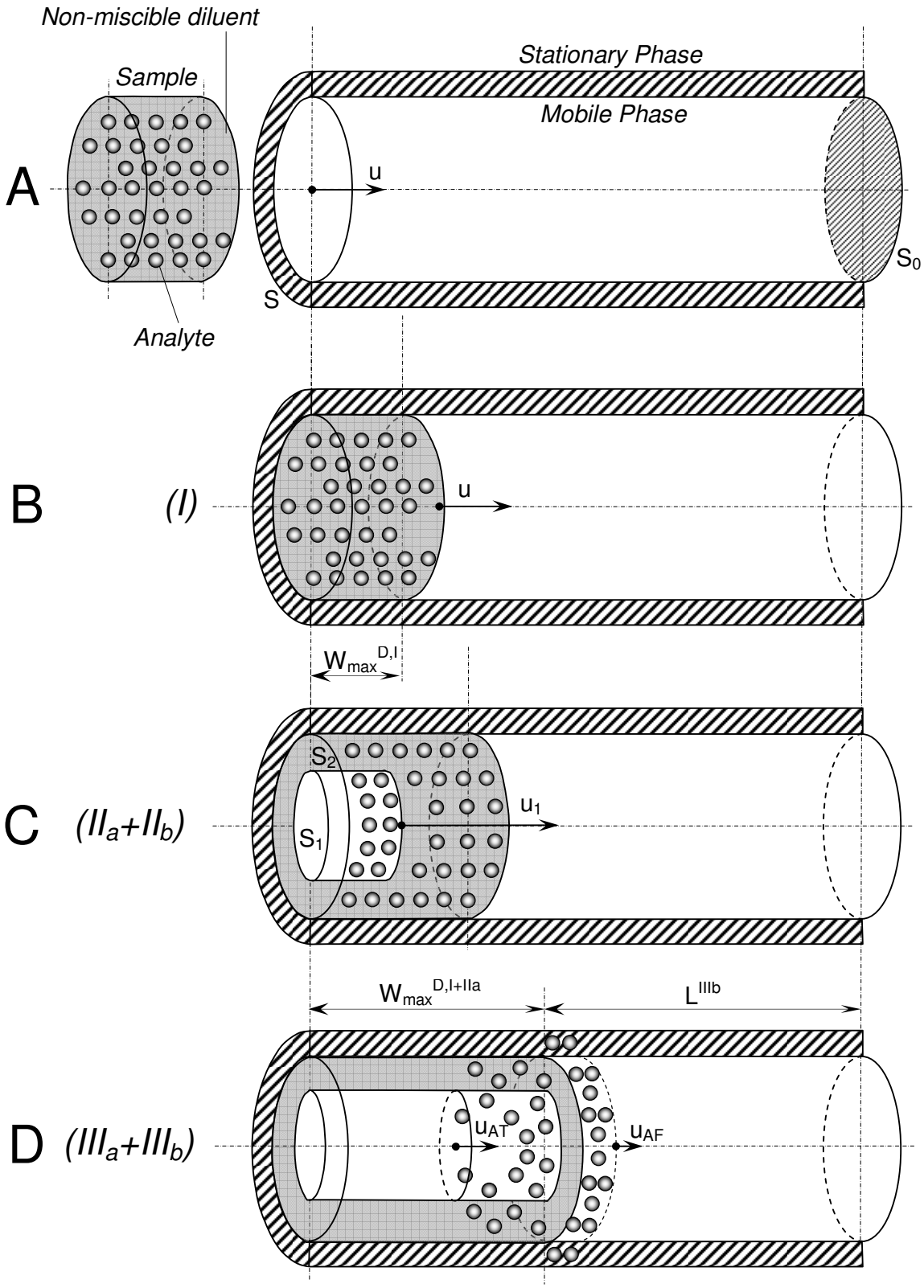

Figure 1

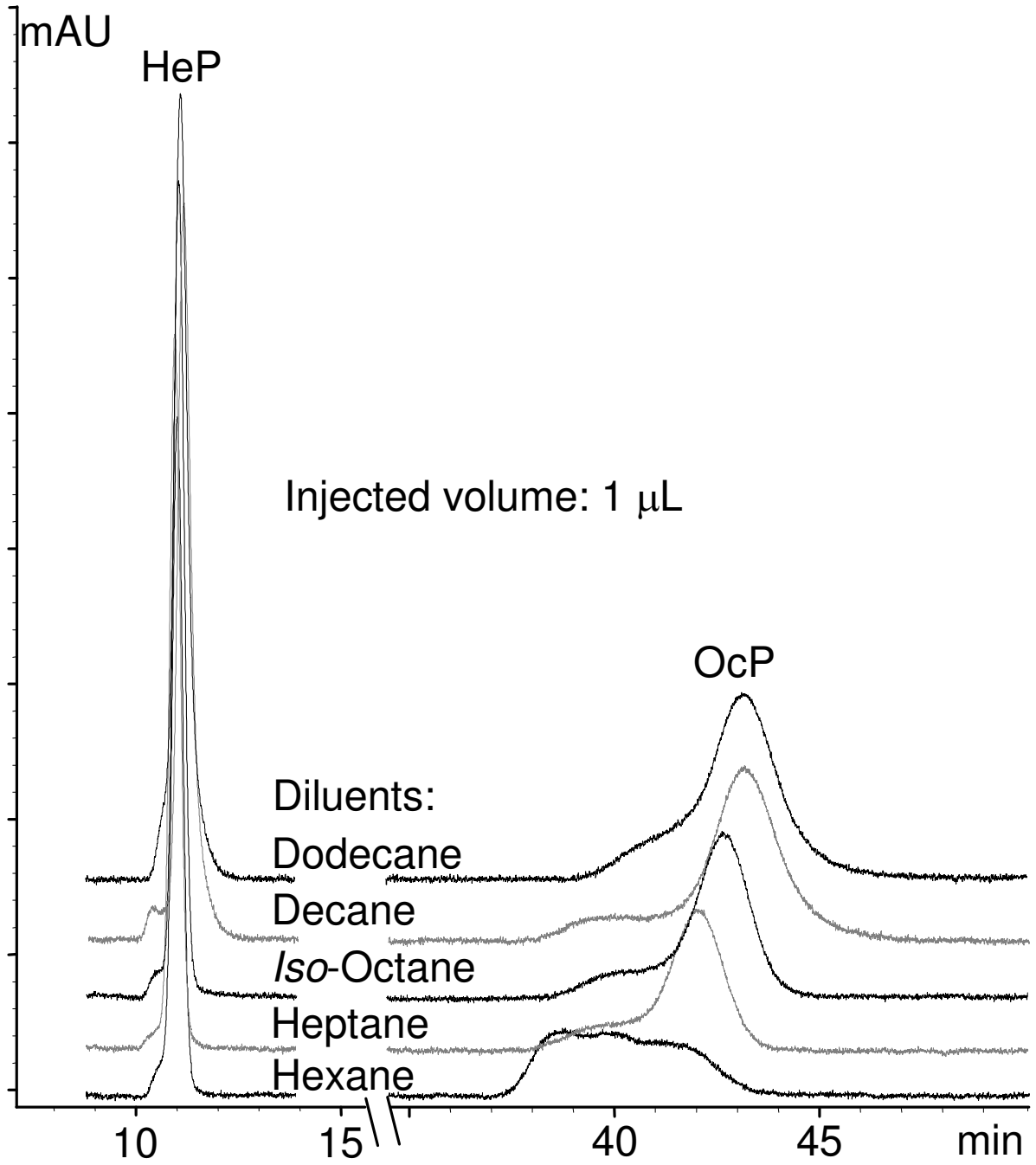

Figure 2

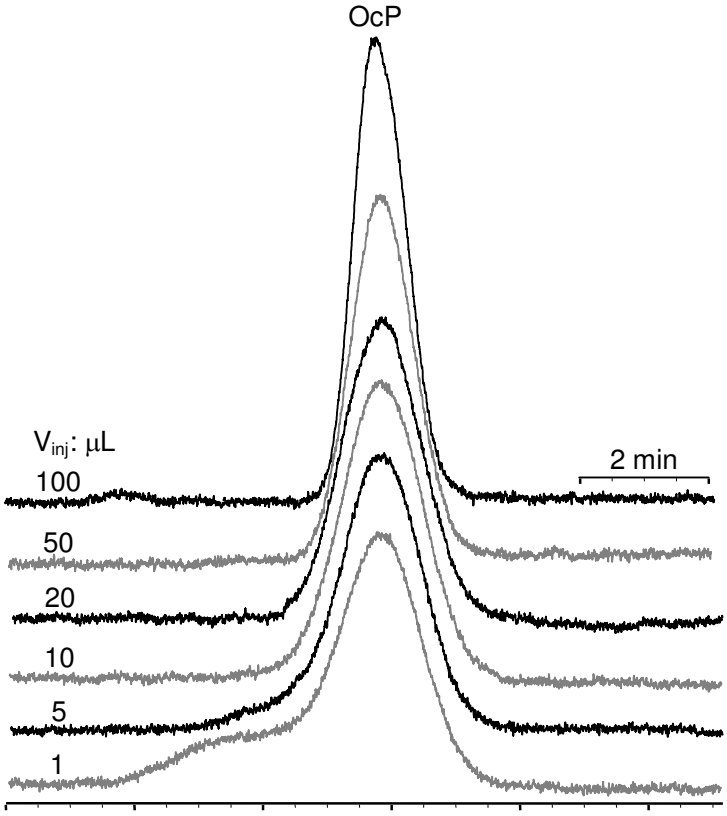

Figure 3

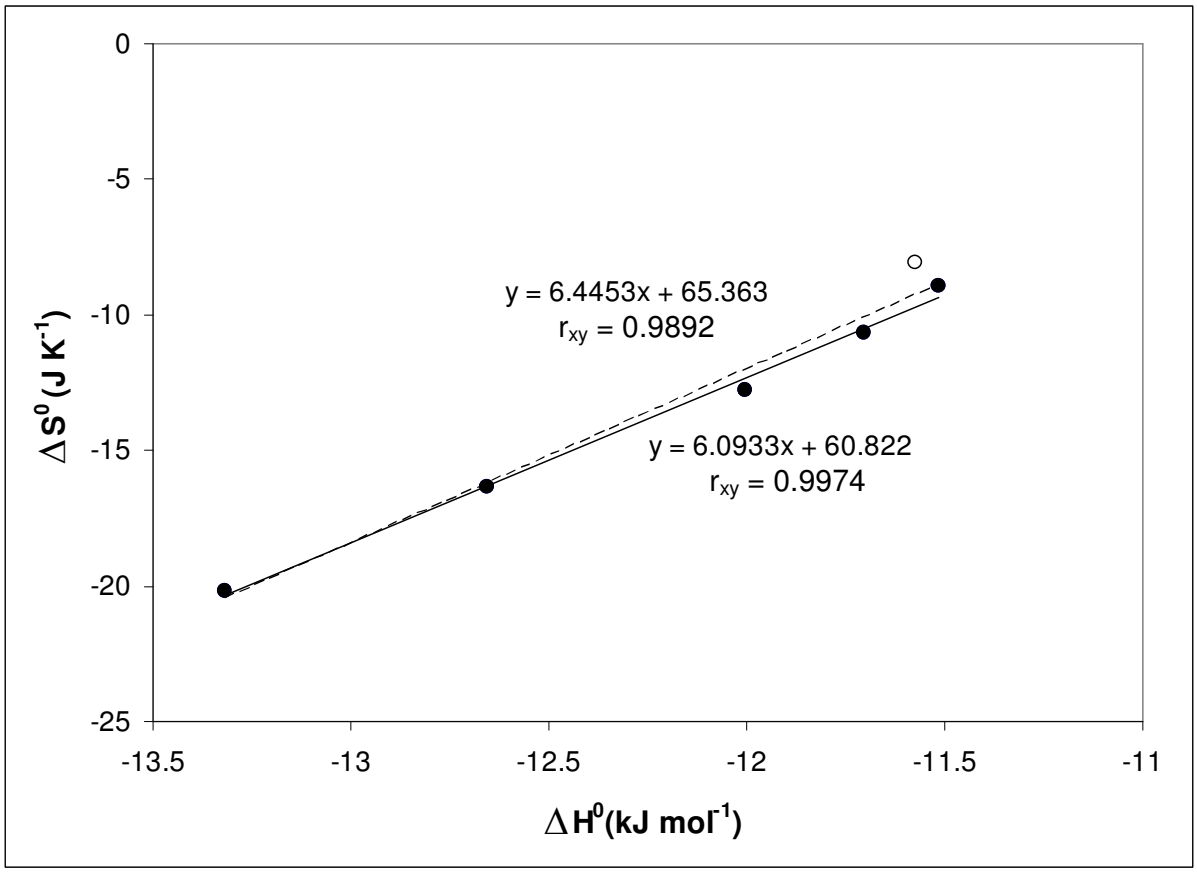

Figure 4

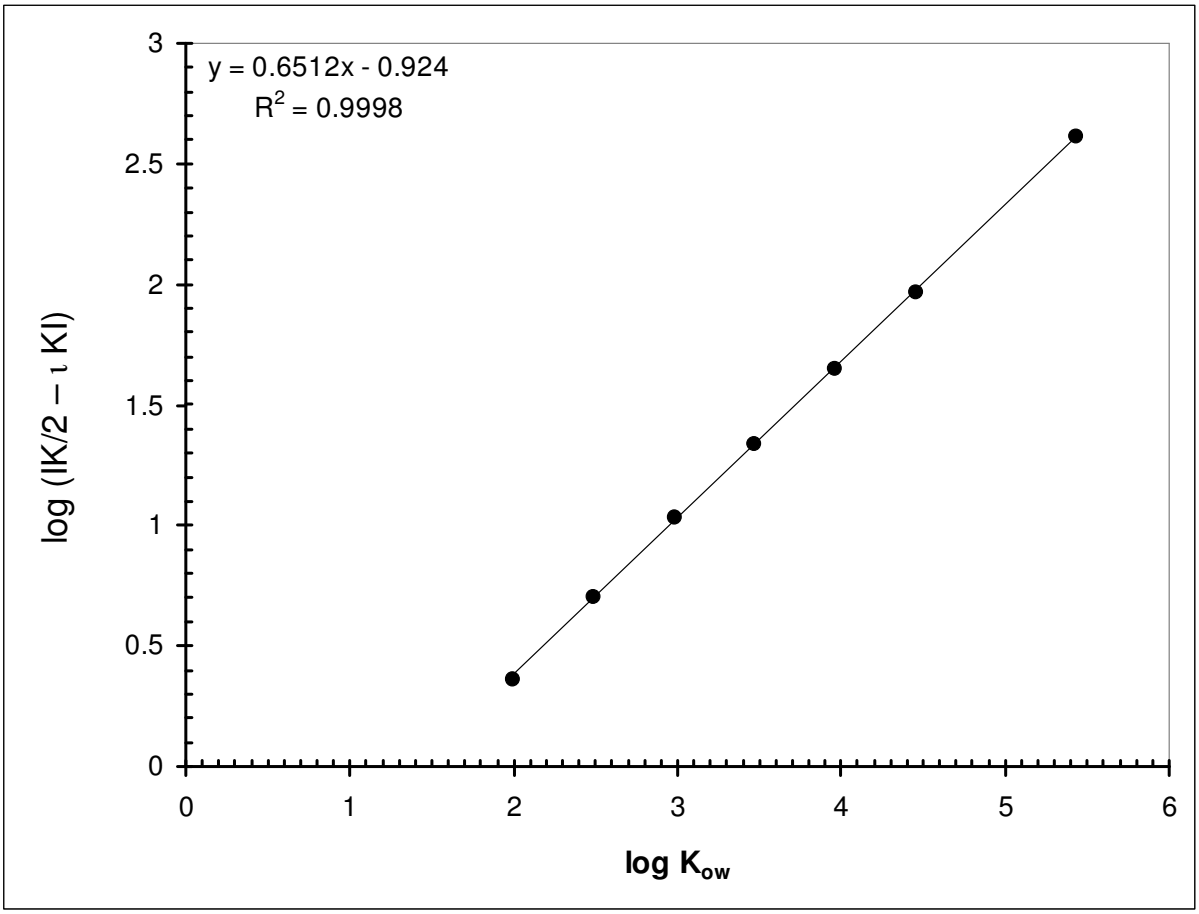

Figure 5

Captions to figures

Figure 1. Steps of the on-line reversed phase supported liquid extraction / liquid chromatography (RP-SLE/LC) on injection of large volumes of immiscible diluents.

Figure 2. Peak shapes corresponding to 1 µL injection volumes of *para*-hexyl (HeP) and *para*-octyl (OcP) hydroxybenzoates in liquid alkanes (hexane, heptane, *iso*-octane, decane, dodecane). Conditions are given in the Experimental section.

Figure 3. Peak shapes of *para*-octyl (OcP) hydroxybenzoate on injection of progressive volumes (1, 5, 10, 20, 50, 100 µL) in heptane.

Figure 4. Enthalpy/entropy compensation plot resulting on injection of increasing volumes (20 up to 100 µL) of the analyte dissolved in heptane (closed circles). The open circle corresponds to the condition corresponding to injection of 1 µL of the methanol solution.

Figure 5. Functional relationship established between the absolute values of the slopes of the linear regressions relating the apparent retention factor (k_{app}) to the reduced injected volume ($V_{inj}\ V_0^{-1}$) and the log K_{ow} of the studied compounds.