Chasing the elusive hold-up time from an LFER approach

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Abstract

A homologous series approach derived from the Abraham's solvation model was developed for the determination of hold-up times. Firstly, it was tested from reversed-phase liquid chromatography data obtained in the literature involving several series of homologues, followed by its application in a polymeric zwitterionic HILIC column using two different homologous series (*n*-alkyl benzenes and *n*-alkyl phenones). Acetonitrile and methanol were selected as organic modifiers in a composition range between 80% and 100% in volume. Results obtained for both series were consistent, and hold-up times were found to be strongly dependent on the water content and the organic modifier nature of the mobile phase.

Highlights

In HILIC hold-up times (or volumes) strongly depend on the mobile phase composition.

A homologous series approach derived from Abraham's solvation model is proposed.

The robustness of the method is assayed with several series of homologues.

Keywords

HILIC, hold-up time, homologous series, void volume

1. Accurate measurement of retention factors

1.1 Importance of hold-up time in the measurement of retention factors

The retention factor, also known as capacity factor, is a fundamental parameter not only for the comparison of chromatographic data obtained from different instruments, but also for the interpretation of physical phenomena taking place inside a column. As discussed later in this paper, in common practice the *k* value of a solute is calculated from its elution time and that expected for a non-retained marker (hold-up time). However, there is not a standard method for the determination of hold-up times covering all chromatographic modes (reversed, normal or HILIC), because the selected approach should depend not only on the particular SP but also on the MP composition (content and nature of the organic modifier, salts…). Moreover, the hold-up time measurement is specially challenging when the boundary between SP and MP is not clearly defined or depends on the MP composition, which is the expected case in HILIC.

The retention factor represents the ratio of the amounts of a solute in the stationary (SP) and mobile (MP) phases, respectively:

$$k = \frac{C_{SP} V_{SP}}{C_{MP} V_{MP}} = \frac{K V_{SP}}{V_{MP}} \quad (1)$$

where C_{SP} and C_{MP} refer to solute concentrations, V_{SP} and V_{MP} are the volumes of SP and MP in the chromatographic column, and K is the equilibrium constant for the distribution of the solute between both phases. A proper and accurate measurement of retention is fundamental in retention modeling [1,2] and the determination of thermodynamic and kinetic parameters related to chromatographic retention [3,4]. In drug discovery LC is an important tool for lead optimization, because the dynamic equilibria of solutes between mobile and SPs might provide biomimetic models accounting for the distribution of compounds between plasma/blood and tissue (lipophilicity, protein and phospholipid binding) [5].

1.2 Contributions to gross retention time and retention factors

IUPAC defines the hold-up volume (time) in column chromatography as “the volume of the mobile phase (or the corresponding time) required to elute a component the concentration of which in the SP is negligible compared to that in the MP. In other words, this component is not retained at all by the SP. Thus, the hold-up volume (time) is equal to the retention volume (time) of an unretained compound. Usually, the hold-up volume (time) includes any volumes contributed by the sample injector, the detector, and connectors” [6]. However, this extracolumn volume depends on the particular chromatographic instrument used (injector, detector cell, tubing -length and internal diameter-, connector fittings…) and it is therefore highly recommendable to measure this

extracolumn contribution to produce chromatographic data independent of the apparatus employed. After subtracting the extracolumn effects from the hold-up volume, the void volume of the column is obtained, i.e. the volume occupied by the MP in the column.

In order to make explicit the different contributions to retention and avoiding the influence of the particular apparatus used, the following expressions seem more adequate than the IUAPC definition above mentioned [7]:

$$t_R = t_R^g - t_{excol} = t_R^{'} + t_M \quad (2)$$

where t_R is the retention time of a compound, t_R^g is the retention time observed on the chromatogram from injection point to detector (gross retention time), t_{excol} is the extracolumn contribution, t_M is the elution time inside the column of an unretained compound (hold-up time), and $t_R^{'}$ is the additional time a molecule is delayed due to its interactions with the SP (adjusted retention time). Since $t_R^{'} = 0$ for an unretained compound, t_M can be expressed as:

$$t_M = t_M^g - t_{excol} \quad (3)$$

where t_M^g is the gross hold-up time. Therefore, for a particular chromatographic instrument and column for which t_{excol} and t_M remain constant, the retention factor (k) is defined as:

$$k = \frac{t_R^{'}}{t_M} = \frac{t_R - t_M}{t_M} = \frac{t_R^g - t_M^g}{t_M^g - t_{excol}} \quad (4)$$

The retention factor expresses how much longer a sample component is retarded by the stationary phase than it would take to travel through the column with the velocity of the MP. k values can be measured from retention volumes as well, since times can be easily converted into volumes by means of the MP flow rate.

1.2 Measurement of hold-up times

There are several methods reported in the literature for the determination of hold-up times [8,9]: pycnometry, homologous series, minor disturbance of baseline, unretained neutral marker, and inorganic salts. In the pycnometric method the column is filled with two solvents of sufficiently different densities and hold-up volumes are calculated from weigh difference. However, this static approach only provide an upper limit to the hold-up volume of the column, since it ignores the possibility of the solvation of the SP by part of the MP. In the homologous series several compounds of the same family (*n*-alkylbenzenes, *n*-alkylalcohols…) are plotted versus their corresponding carbon number, and the hold-up time is calculated from regression analysis. According to the minor disturbance method, the hold-up time is determined from the baseline disturbance caused by a MP component, normally deuterated in order to detect a reliable disturbance peak. Because of its ease of

use, UV absorbing neutral molecules, non-polar for normal phase (tetrachloromethane, benzene, toluene…) and polar for reversed phase (formamide, phloroglucinol, thiourea, uracil, urea…) were proposed as suitable unretained markers. However, in reversed phase they are likely to show partial retention and inorganic salts are preferred instead ($NaNO_3$, KBr…), with the caution of using eluents containing buffers or other salts in order to avoid the exclusion of anions from the pores of the SP due to residual silanol groups.

Due to the complexity of separation mechanisms in hydrophilic interaction chromatography (HILIC), the selection of a suitable hold-up time marker is challenging. It is assumed that the main retention mechanism is based on the partition of solutes between the bulk MP and a water enriched layer that is semi-immobilized on the SP. Thus, non-polar hydrophobic substances appear to be suitable markers. McCalley and Neue [10] studied the retention of benzene and toluene in different silica columns using acetonitrile/water MPs, and they found that retention depended on the fraction of water in the eluent. Under typical HILIC conditions (up to 30% of water) retention times decreased with the thickness of water layer on the silica surface, and consequently the retention volume of marker was lower than the void volume calculated by pycnometry. Therefore, the column void volume greatly depends on the boundary between stationary and MPs, and this greatly depends on the column employed but also on the particular eluent composition. Concerning the column, the functionalization of the SP is a key factor on chromatographic retention and selectivity, but the interactive layer between the silica support and functionality also plays an important role [11]. Even with the same functionality, a polymer grafted silica lead to higher water uptake capacities than silicas functionalized through conventional silane chemistry [12].

1.3 Homologous series: an LFER approach

The homologous series approach for hold-up time determination presented in the present work is based on linear free energy relationships (LFER), particularly on the solvation model proposed by Abraham [13]. In this case the solvation property, log k, is linearly related to specific interactions between solute and surrounding phase, mainly dispersion ($e \cdot E$), dipole-dipole or dipole-induced dipole plus some polarizability interactions ($s \cdot S$), solute hydrogen-bond acidity and basicity ($a \cdot A$ and $b \cdot B$, respectively), and a volume term ($v \cdot V$) related to the endoergic work of separating solvent molecules to provide a cavity of suitable size for the solute molecule and the exoergic solute-solvent general dispersion interactions:

$$\log k = c + eE + sS + aA + bB + vV \tag{5}$$

where E, S, A, B, and V are solute descriptors, and e, s, a, b, and v are the system constants.

Combining Eqs. (4) and (5) we obtain a general expression for the retention time of a solute:

$$t_R = t_M + t_M 10^{c + eE + sS + aA + bB + vV} \tag{6}$$

As shown by the molecular descriptors *E*, *S*, *A*, and *B* presented in Table 1, the compounds forming part of a homologous series share very similar dispersive, dipolarity/polarizability, and hydrogen-bonding features, only differing in the number of carbon atoms (n_C) and consequently in the molecular volume (*V*). In relation to the chromatographic conditions, as long as there is no variation in both the SP and the MP, the system coefficients *c*, *e*, *s*, *a*, and *b* remain unchanged throughout the analysis of the whole series. Therefore, $c+eE+sS+aA+bB$ should be a constant value for all solutes in the series, and consequently $t_M 10^{c+eE+sS+aA+bB}$ should be a constant value (*r*) as well for the homologous series and chromatographic system under consideration. Thus, Eq. (6) for the components of a homologous series can be expressed as:

$$t_R = t_M + r10^{vV} \tag{7}$$

where *r* and *v* are constant values. *V* is the McGowan characteristic volume of the solute but divided by 100 in order to have similar values to the other molecular descriptors (units of mL $mol^{-1}/100$). The McGowan volume for a particular solute can be easily calculated as the sum of the characteristic volumes of its atoms, subtracting 6.56 cm^3 mol^{-1} for each bond [14]. For complicated molecules, the number of bonds can be obtained from the total number of atoms (N_a) and the number of rings (R_g) [13]. Thus, *V* can be calculated as:

$$V = \frac{\sum(\text{all atom contributions}) - 6.56(N_a - 1 + R_g)}{100} \tag{8}$$

For instance, the characteristic atomic volumes of C, N, O, F, Cl, Br, and H are 16.35, 14.39, 12.43, 10.48, 20.95, 26.21, and 8.71 mL mol^{-1}, respectively.

All the molecular descriptors mentioned (*E*, *S*, *A*, *B*, and *V*) can be directly obtained, either experimentally determined or calculated, from paid [15] or open access [16] databases.

In fact, Eq. (8) is similar to the expression describing the retention times of homologous series [17]:

$$t_R = t_M + e^{c_0 + c_1 \cdot n_C} \tag{9}$$

where c_0 and c_1 are constant parameters depending on the particular homologous series used and the chromatographic conditions (column and MP), and n_C is the number representing the size of the homologous unit, usually the number of carbon atoms of this unit. In mostly of the cases, hold-up times can either be obtained from Eqs. (7) or (9) with the same figures (t_M value and corresponding standard error, determination coefficient…) from fitting statistics. However, the LFER approach proposed in this work implies a previous study of the candidate compounds by means of their molecular descriptors in order to check whether they form a reliable homologous series and justifies the fitting parameters from the solute-solvent interactions.

2. Materials and methods

HPLC measurements were performed on a Shimadzu (Kyoto, Japan) HPLC system consisting of two LC-10ADvp pumps, a SIL-10ADvp auto-injector, an SPD-M10AVvp diode array detector and a CTO-10ASvp oven at 25 °C and a SCL-10Avp controller. A 5 μm, 150 x 4.6 mm ZIC-pHiLIC (Merck, Darmstadt, Germany) column was employed. Injection volume and flow rate were 1 μL and 0.50 mL min^{-1}, respectively.

Extracolumn volume was determined injecting 1 μL of 0.4 mg mL^{-1} aqueous solution of potassium bromide (Merck, >99%) in absence of column and using water as eluent at two different flow rates, 0.25 and 0.50 mL min^{-1}, each in triplicate. The overall extracolumn volume in the particular chromatograph employed was 0.101(0.003) mL.

pH was measured using a Crison 5014 combined electrode connected to a GLP 22 potentiometer from Crison (Barcelona, Spain), and standard aqueous solutions (pH 4 and 7) were used for calibration.

Water was obtained from a Milli-Q plus system (Millipore, Billerica, USA) with a resistivity of 18.2 MΩ cm. Aqueous buffer was directly prepared from ammonium acetate (Sigma-Aldrich, Baker, (≥ 98%) at different concentrations in order to provide a total concentration in the MP of 5 mM. The organic modifiers used in HPLC MPs were acetonitrile and methanol (Fisher, HPLC gradient grade). The injected *n*-alkyl benzenes and phenones were purchased from Acros Organics, Alfa Aesar, Merck, and Sigma-Aldrich; all of high purity grade (≥ 97%).

Stock solutions of injected analytes were prepared in methanol at a concentration of 5 mg mL^{-1}, and diluted to 1 mg mL^{-1} before injection.

3. Results and discussion

3.1 Testing the LFER homologous series approach from RP literature data

In 2002 Rimmer *et al.* [9] published and excellent review about the measurement and meaning of void volumes in reversed-phase (RP) liquid chromatography, including a section dealing with the homologous series approach and pointing out the advantages and limitations. This method was traditionally based on the linear relationship obtained in the series plot of the logarithm of the adjusted retention time ($\log t'_R = \log(t_R - t_M)$) versus the homolog number (n_C). However, the number of homologues included in the series, their size or nature were considered as critical parameters in RP. The multiparametric nonlinear regression approach (Eq. (9)) was later introduced leading to shorter calculation times with results in good agreement with those resulting from linear methods [18].

In order to test the LFER homologous series approach developed in this work, data presented in literature has been fitted to Eq. (7). An *n*-alkyl alcohol homologous series (Table 1) was chosen by

Nowotnik and coworkers for the measurement of hold-up times in RP using a polystyrene divinylbenzene SP and a MP containing a 65% of acetonitrile (ACN). The plot of retention versus McGowan volumes (Figure 1) shows the expected profile of a RP mode: the higher the molecular volume, the higher the retention. Fitting statistics were excellent (Table 2) and the hold-up volume presents a value of 1.22(±0.01) mL, which is in very good agreement with the volumes directly measured from deuterated water and sodium nitrate markers, both reported to be 1.26 mL. Figure 1 also shows the data from Shibukawa *et al.* [19], who used the same homologous series in a 40% ACN/water MP, but this time with a C18 SP. Excellent fitting figures were also obtained (Table 2), with a good match between the fitted hold-up volume and that directly measured from nitrate injection (1.54(±0.05) and 1.53 mL, respectively).

Montes *et al.* published in 1989 [20] a very interesting study involving non-linear *N*-nitrosamine series (Table 1), a normal-phase propyl cyano SP and MPs containing methanol (MeOH) and ACN as organic modifiers. Since both the MP and the SP were polar one would think about HILIC mode, but as presented in Figure 1 it was not the case. The larger the solute and the water content in the MP, the larger the retention, showing a typical reversed-phase behavior. The relatively low amount of organic solvent, up to 60% for MeOH and 50% for ACN, was probably not high enough for HILIC. Notice that nearly the same retention volumes were reported for the two pairs of homologues with the same McGowan molecular volume (or n_C), those with n_C=4 and n_C=6. In all cases experimental could be nicely fitted to Eq. (7) (Table 2).

The characterization of RP systems by means of the Abraham's solvation parameter (Eq. (5)) shows that solute volume is the main factor increasing the retention [24], which indicates that the rearrangements of the solvent molecules in the hydroorganic MP to create a cavity for the solute is more energetically demanding than the analogous process in the non-polar SP. Consequently, as expected for a RP mode, the *v* system coefficients reported in Table 2 were positive. Moreover, notice the decrease of *v* value when the content of MeOH or ACN in the mobile phase increases, suggesting that for high content of organic solvent *v* might be negative and thus the system would work in HILIC mode.

Contrarily to the good fittings observed for *n*-alkyl alcohols and *N*-nitrosamines for RP using hydroorganic MPs containing MeOH or ACN, worse figures were obtained in the case *n*-alkyl benzenes or when THF was used as organic modifier (data from refs. [21,22] and [23], respectively, results not shown).

3.2 Testing the LFER homologous series approach in HILIC

On the one hand, the ZIC-pHILIC column employed in the present work is functionalized by a polymer layer carrying one sulfobetaine moiety for each monomeric unit, and this polymeric SP is expected to form a hydrogel layer [11]. The SP water uptake is related to the water content in the MP, and this hydrogel layer becomes thicker until the maximum extension of the grafted chains due to water swelling is reached [12]. Therefore, the hold-up volum in this column was expected to be very sensitive to the water content in the MP. On the other hand, two different homologous series were selected in this study, *n*-alkyl benzenes and *n*-alkyl phenones (Table 1). Although both series share in common a null hydrogen-bonding donor capacity, *n*-alkylphenones are significantly more polar (*S*), better hydrogen-bonding acceptors (*B*) and slightly more polarizable (*E*), Since the most common detection in liquid chromatography is UV absorbance, homologues containing aromatic rings are preferred than those requiring other more expensive or less widespread used instrumentation, as it would be the case of refractive index detector for alkyl alcohols.

Figure 2 shows the plots obtained for both series of homologues using hydroorganic MP in the range between 80% and 100% of ACN or MeOH. In contrast to the typical RP behavior (Figure 1), in HILIC and for both organic solvents the retention decreases with the molecular volume of the homologue. However, the retention behavior depends on the organic solvent used in the MP. In the case of ACN the retention decreases with the water content increase, whereas for MeOH the reverse trend is observed. Generally, retention times are well described by the model proposed in this work, with determination coefficients close to 1 (Table 3). In contrast to RP, fitted *v* values were negative, suggesting that in HILIC more energy is required to form a cavity in the highly cohesive aqueous layer adsorbed on the SP than in the hydroorganic MP.

Frequently toluene is used as hold-up marker in HILIC. However, this chromatographic practice should be examined with caution due to the significant differences obtained in this work between the retention times of toluene and the fitted hold-up volumes. As a representative example, Table 4 shows a list of compounds less retained than toluene in mobile phases containing a 90% of acetonitrile or methanol. With a much larger molecular volume, the retention times obtained from dodecylbenzene as hold-up time marker are closer to those obtained from the LFER approach.

The fitted hold-up times presented in Table 3 were converted into volumes by means of the MP flow rate and they are reported in Table 5, obtaining slightly lower times for the alkylbenzenes in relation to alkylphenones. Although differences between both series of homologues are statistically significant due to the reduced figures of the standard errors of the fitted void volumes, from a chromatographic point of view the results obtained for both families of compounds can be considered very similar (differences are in all cases below 5%). Regarding ACN as MP component, the reduction of hold-up volume with the water content is consistent with the formation of the hydrogel layer

mentioned at the beginning of this section. The higher the water content in the MP, the thicker the water layer adsorbed on the SP and consequently the lower the volume of mobile solvent inside the column. This explanation assumes that ACN is not wetting at all (or at the most very poorly) the SP. However, further research is necessary to explain the relationship between retention and MeOH content in the MP. According to Kamlet and Taft's solvatochromic parameters [25], pure MeOH and ACN are solvents of similar polarity/dipolarizability (π^*, 0.60 and 0.66, respectively), but presenting the alcohol a better hydrogen-bond acceptor basicity (β, 0.66 and 0.40) and a significantly higher hydrogen-bond donor acidity (α, 0.98 and 0.19), being the latter more similar to that of water (1.17). In fact, it was reported in literature [26] that the mixtures of MeOH and water might lead to a ternary system consisting of associated water molecules, associated MeOH, and water associated with MeOH, the three of them behaving differently in relation to solute distribution. Therefore, the swelling of the SP with both MeOH and water mixtures might be possible, or even their adsorption on the SP leading to a partition mechanism of the solutes between the immobilized hydroorganic layer and the bulk MP.

With the aim of studying the effect of the number of homologues included in correlations on the fitted hold-up times, the largest or the smallest individuals in the series were sequentially excluded from the complete set of 9 substances (8 in the case of alkyl phenones at 100% of organic solvent). As illustrated in Figure 3 when ACN was used in the MP, in the alkyl phenone series the fitted hold-up times remain unchanged in both scenarios for all the studied MP compositions. Similar results were obtained for alkyl benzenes, with the exception of 80% and 100% of ACN when octyl- and heptylbenzene (N=7) together with hexylbenzene (N=6) were excluded. In the case of MeOH (Figure 4) the associated standard errors were generally higher and major variations were found when aceto- and propiophenone (N=7) together with butyrophenone (N=6) were taken out. However, variations in hold-up times are in general quite contained, suggesting a good robustness of the model proposed.

4. Conclusions

A LFER method based on the retention of homologous series has been developed for the determination of hold-up times, which was tested in both RP and HILIC. This approach not only leads to accurate determination of hold-up times and volumes, but also provides information of the predominant mode of retention (RP or HILIC). In RP the retention plot increases with the molecular volume of the homologue, whereas in HILIC it decreases.

In HILIC hold-up times strongly depend on the MP composition, particularly the water content and the organic modifier employed. The homologous series approach followed, derived from the Abraham's solvation model, led to the determination of very similar hold-up times from *n*-alkyl

benzene and *n*-alkyl phenone series in a polymeric zwitterionic ZIC-pHILIC column. When ACN was used as MP component hold-up times increased with the organic modifier content, whereas for MeOH the reverse trend was observed. This fact suggests that the fixed solvent layer on the SP is mostly composed of water when using ACN/water MPs, whereas it would be constituted of both MeOH and water molecules in the case of MeOH/water MPs.

The fitted times obtained from *n*-alkyl phenone series in ACN/water MP were unaffected by the reduction of the largest or smallest homologues.

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Conflict of interest statement

The authors declare no conflict of interest.

TABLES

Table 1. Molecular descriptors of the homologous series considered in this work [16].

Homologous series	n_C	E	S	A	B	V
n-Alkyl alcohols						
Methanol	1	0.28	0.44	0.43	0.47	0.308
Ethanol	2	0.25	0.42	0.37	0.48	0.449
Propanol	3	0.24	0.42	0.37	0.48	0.590
Butanol	4	0.22	0.42	0.37	0.48	0.731
Pentanol	5	0.22	0.42	0.37	0.48	0.872
Hexanol	6	0.21	0.42	0.37	0.48	1.013
Heptanol	7	0.21	0.42	0.37	0.48	1.154
Octanol	8	0.20	0.42	0.37	0.48	1.295
N-nitrosamines						
N-Nitrosodimethylamine	2	0.39	1.19	0.00	0.56	0.606
N-Nitrosomethylethylamine	3	0.37	1.10	0.00	0.59	0.747
N-Nitrosodiethylamine	4	0.35	1.08	0.00	0.59	0.888
N-Nitrosomethylpropylamine	4	0.36	1.05	0.00	0.60	0.888
N-Nitrosomethylbutylamine	5	0.34	1.07	0.00	0.60	1.028
N-Nitrosodipropylamine	6	0.32	1.01	0.00	0.63	1.169
N-Nitrosoethylbutylamine	6	0.32	1.00	0.00	0.62	1.169
N-Nitrosopropylbutylamine	7	0.31	1.02	0.00	0.63	1.310
N-Nitrosodibutylamine	8	0.30	0.98	0.00	0.64	1.451
n-Alkyl benzenes						
Benzene	0	0.61	0.52	0.00	0.14	0.716
Toluene	1	0.60	0.52	0.00	0.14	0.857
Ethylbenzene	2	0.61	0.51	0.00	0.15	0.998
Propylbenzene	3	0.60	0.50	0.00	0.15	1.139
Butylbenzene	4	0.60	0.51	0.00	0.15	1.280
Pentylbenzene	5	0.59	0.51	0.00	0.15	1.421
Hexylbenzene	6	0.59	0.50	0.00	0.15	1.562
Octylbenzene	8	0.58	0.48	0.00	0.15	1.844
Dodecylbenzene	12	0.57	0.47	0.00	0.15	2.407
n-Alkyl phenones						
Acetophenone	2	0.82	1.01	0.00	0.48	1.014
Propiophenone	3	0.80	0.95	0.00	0.51	1.155
Butyrophenone	4	0.80	0.95	0.00	0.51	1.296
Valerophenone	5	0.80	0.95	0.00	0.50	1.437
Hexanophenone	6	0.78	0.95	0.00	0.51	1.578
Heptanophenone	7	0.77	0.95	0.00	0.50	1.718
Octanophenone	8	0.77	0.95	0.00	0.50	1.859
Nonanophenone	9	0.76	0.95	0.00	0.50	2.000
Decanophenone	10	0.75	0.95	0.00	0.50	2.141

Table 2. Fitted t_M, r, and v parametres (Eq. (7)) from *n*-alkyl alcohol and *N*-nitrosamine homologous series from literature date in RP (standard errors of the fitted parameters in brackets). Number of homologues (N) used in the fittings and the coefficient of determination (R^2) are also reported.

Homologues	SP	MP	t_M (min)	r	v	R^2	N
n-Alkyl alcohols	PS-DVD	65% ACN	1.22(0.01)	0.08(0.01)	1.11(0.02)	0.9999	8
	C18	40% ACN	1.54(0.05)	0.08(0.01)	1.56(0.03)	0.9998	8
N-Nitrosamines	Propyl ciano	55% ACN	2.59(0.06)	0.28(0.05)	0.40(0.03)	0.9995	9
		50% ACN	2.45(0.06)	0.36(0.04)	0.44(0.03)	0.9997	9
		45% ACN	2.56(0.03)	0.27(0.02)	0.60(0.01)	0.9999	9
		40% ACN	2.60(0.04)	0.23(0.02)	0.72(0.02)	0.9998	9
		35% ACN	2.71(0.05)	0.16(0.02)	0.91(0.03)	0.9996	9
		30% ACN	2.83(0.05)	0.10(0.01)	1.12(0.03)	0.9996	9
		25% ACN	2.99(0.07)	0.06(0.01)	1.40(0.04)	0.9994	9
		20% ACN	3.12(0.09)	0.04(0.01)	1.66(0.05)	0.9994	9
		60% MeOH	2.88(0.03)	0.14(0.02)	0.59(0.03)	0.9995	9
		55% MeOH	2.90(0.03)	0.12(0.01)	0.77(0.02)	0.9997	9
		50% MeOH	2.97(0.06)	0.09(0.02)	0.99(0.05)	0.9989	9
		45% MeOH	2.91(0.05)	0.10(0.01)	1.08(0.03)	0.9996	9
		40% MeOH	3.01(0.05)	0.07(0.01)	1.32(0.03)	0.9996	9
		35% MeOH	3.07(0.08)	0.05(0.01)	1.53(0.04)	0.9995	9
		30% MeOH	3.15(0.10)	0.04(0.01)	1.77(0.03)	0.9997	9

Table 3. Fitted t_M, r, and v parametres (Eq. (7)) from *n*-alkyl benzene and *n*-alkyl phenone homologous series for each studied MP composition in HILIC (standard errors of the fitted parameters in brackets). Each compound was injected in triplicate. Number of homologues (N) used in the fittings and the coefficient of determination (R^2) are also reported.

Homologous series	MP	t_M (min)	r	v	R^2	N
n-Alkyl benzenes	100% ACN	2.93(0.0204)	1.06(0.08)	-0.46(0.08)	0.9597	9
	95% ACN	2.75(0.01)	1.07(0.03)	-0.61(0.02)	0.9977	9
	90% ACN	2.61(0.01)	1.18(0.04)	-0.67(0.03)	0.9965	9
	85% ACN	2.47(0.01)	1.23(0.03)	-0.64(0.02)	0.9982	9
	80% ACN	2.30(0.03)	1.22(0.06)	-0.50(0.05)	0.9848	9
	100% MeOH	2.89(0.01)	2.22(0.06)	-0.65(0.02)	0.9979	9
	95% MeOH	2.92(0.01)	2.19(0.04)	-0.59(0.02)	0.9985	9
	90% MeOH	3.03(0.02)	2.26(0.08)	-0.57(0.03)	0.9949	9
	85% MeOH	3.21(0.02)	2.17(0.06)	-0.52(0.03)	0.9959	9
	80% MeOH	3.83(0.01)	2.28(0.14)	-0.82(0.04)	0.9926	9
n-Alkyl phenones	100% ACN	3.03(0.01)	2.23(0.25)	-0.69(0.06)	0.9925	8
	95% ACN	2.81(0.01)	1.82(0.15)	-0.68(0.04)	0.9949	9
	90% ACN	2.64(0.01)	2.36(0.24)	-0.84(0.05)	0.9937	9
	85% ACN	2.50(0.01)	2.18(0.10)	-0.79(0.02)	0.9986	9
	80% ACN	2.41(0.01)	2.00(0.09)	-0.69(0.03)	0.9983	9
	100% MeOH	2.99(0.02)	5.16(0.37)	-0.79(0.04)	0.9974	8
	95% MeOH	3.04(0.01)	4.20(0.21)	-0.71(0.03)	0.9982	9
	90% MeOH	3.15(0.02)	3.75(0.24)	-0.70(0.03)	0.9970	9
	85% MeOH	3.33(0.02)	3.26(0.29)	-0.71(0.05)	0.9940	9
	80% MeOH	3.84(0.05)	3.74(2.57)	-0.95(0.32)	0.8073	9

Table 4. Some compounds are less retained than toluene in mobile phases containing acetonitrile or methanol (mean values of three replicates, standard errors in brackets). Hold-up times obtained from *n*-alkyl benzene and *n*-alkyl phenone homologous series are also presented.

90% Acetonitrile		90% Methanol	
Compounds	t_R (min)	Compounds	t_R (min)
Alkyl benzene series	***2.615(0.009)***	***Alkyl benzene series***	***3.026(0.032)***
Alkyl phenone series	***2.637(0.008)***	Dodecylbenzene	3.109(0.008)
Dodecylbenzene	2.638(0.002)	Geraniol	3.122(0.002)
Decanophenone	2.671(0.005)	***Alkyl phenone series***	***3.154(0.026)***
Nonanophenone	2.689(0.005)	α-Pinene	3.243(0.002)
Octylbenzene	2.689(0.006)	Octylbenzene	3.247(0.007)
Octanophenone	2.703(0.008)	Decanophenone	3.273(0.019)
Dodecanophenone	2.728(0.008)	Nonanophenone	3.295(0.004)
Hexylbenzene	2.730(0.002)	Hexylbenzene	3.334(0.011)
Heptanophenone	2.731(0.011)	Octanophenone	3.345(0.004)
Pentylbenzene	2.743(0.013)	Hydrocortisone	3.374(0.010)
Hexanophenone	2.751(0.007)	Phenol	3.380(0.013)
α-Pinene	2.760(0.016)	Resorcinol	3.380(0.009)
Benzyl benzoate	2.779(0.016)	Thiourea	3.386(0.022)
Butylbenzene	2.780(0.002)	Pentylbenzene	3.391(0.008)
Valerophenone	2.788(0.006)	2-Nitroaniline	3.395(0.017)
Propylbenzene	2.814(0.008)	Heptanophenone	3.399(0.006)
Butyrophenone	2.827(0.016)	Dodecanophenone	3.413(0.010)
Ethylbenzene	2.839(0.007)	Hexanophenone	3.429(0.009)
p-Xylene	2.841(0.009)	Butylbenzene	3.436(0.034)
Thymol	2.853(0.011)	Antipyrine	3.472(0.007)
Ethylbenzene	2.864(0.001)	Thymol	3.477(0.011)
Geraniol	2.868(0.012)	Benzaldehyde	3.478(0.007)
Benzophenone	2.884(0.012)	Propylbenzene	3.516(0.006)
Propiophenone	2.891(0.001)	Valerophenone	3.528(0.001)
2-Nitroanisole	2.891(0.012)	Butyrophenone	3.611(0.005)
Benzamide	2.898(0.008)	Ethylbenzene	3.635(0.005)
Methyl benzoate	2.909(0.012)	*p*-Xylene	3.654(0.005)
Benzonitrile	2.930(0.013)	Monuron	3.688(0.071)
Toluene	***2.931(0.004)***	Propiophenone	3.743(0.005)
		Acetanilide	3.754(0.004)
		Toluene	***3.773(0.011)***

Table 5. Fitted hold-up volumes (mL) from Eq. (7) for the studied homologous series for each MP composition (95% confidence interval in brackets).

Organic solvent content	Acetonitrile		Methanol	
	n-Alkyl benzenes	*n*-Alkyl phenones	*n*-Alkyl benzenes	*n*-Alkyl phenones
100%	1.47(±0.04)	1.52(±0.02)	1.45(±0.01)	1.50(±0.02)
95%	1.38(±0.01)	1.40(±0.01)	1.46(±0.01)	1.52(±0.01)
90%	1.31(±0.01)	1.32(±0.01)	1.51(±0.02)	1.58(±0.02)
85%	1.23(±0.01)	1.25(±0.01)	1.60(±0.02)	1.67(±0.02)
80%	1.15(±0.03)	1.20(±0.01)	1.91(±0.01)	1.92(±0.05)

FIGURES

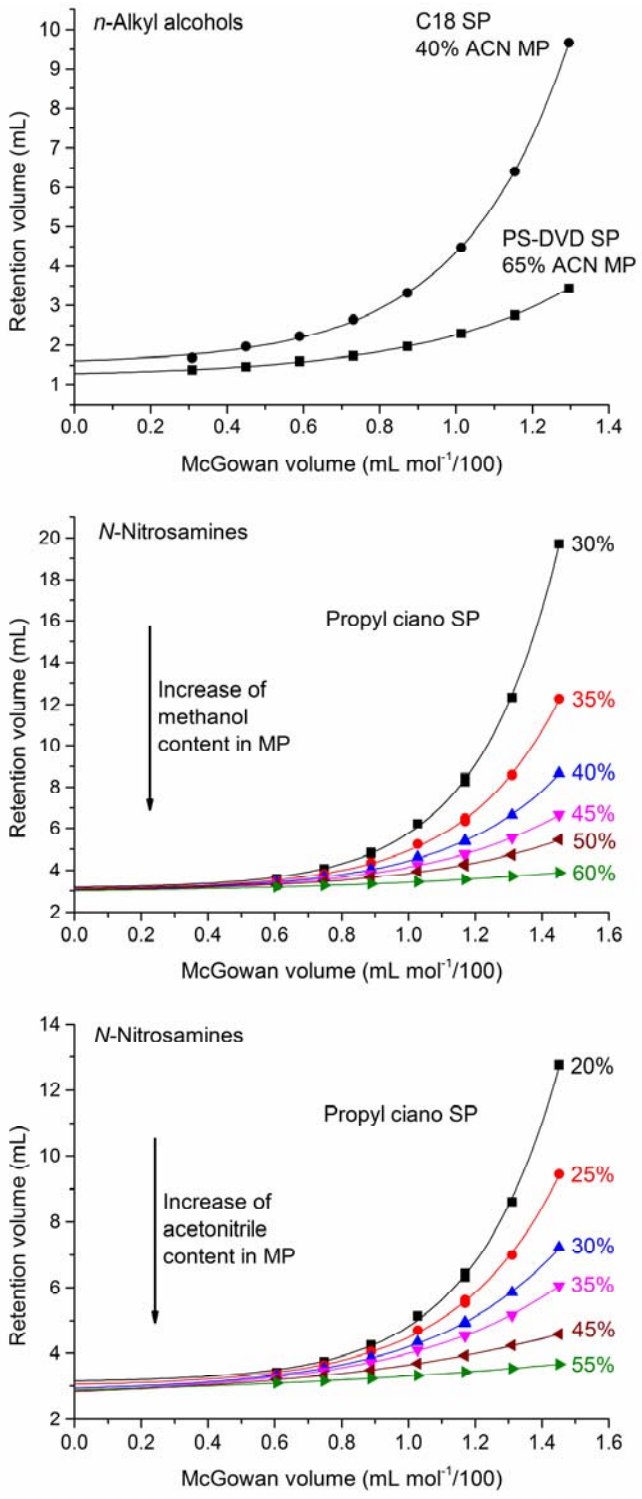

Figure 1. Variation of retention volumes of linear (*n*-alkyl alcohols) and non-linear (*N*-nitrosamines) homologous series with the molecular volume of the individuals and fittings according to Eq. (7).

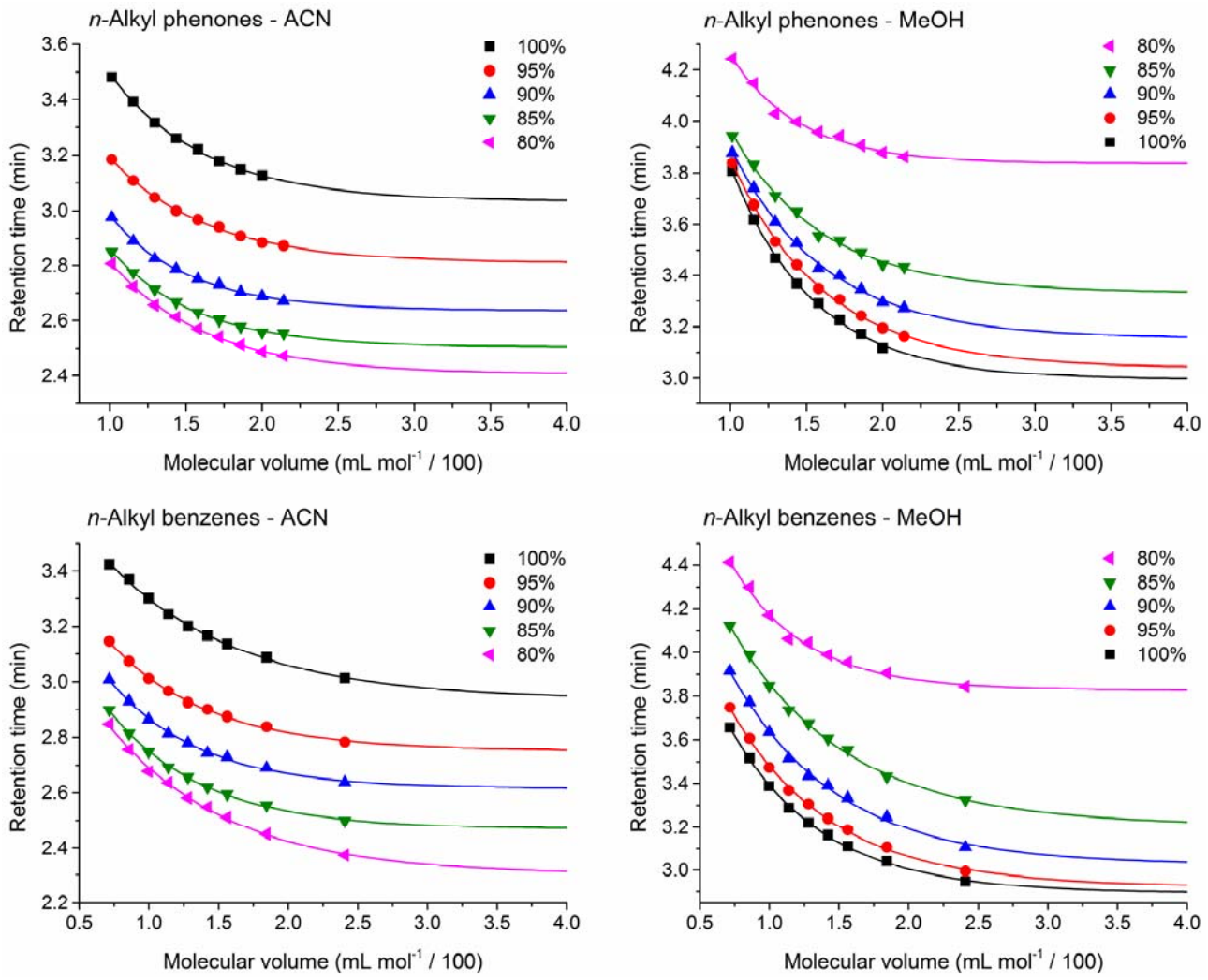

Figure 2. Mean values of retention times of *n*-alkyl benzene and *n*-alkyl phenone series depending on the organic modifier added to the MP (ACN or MeOH). Solid lines show fittings to Eq. (7).

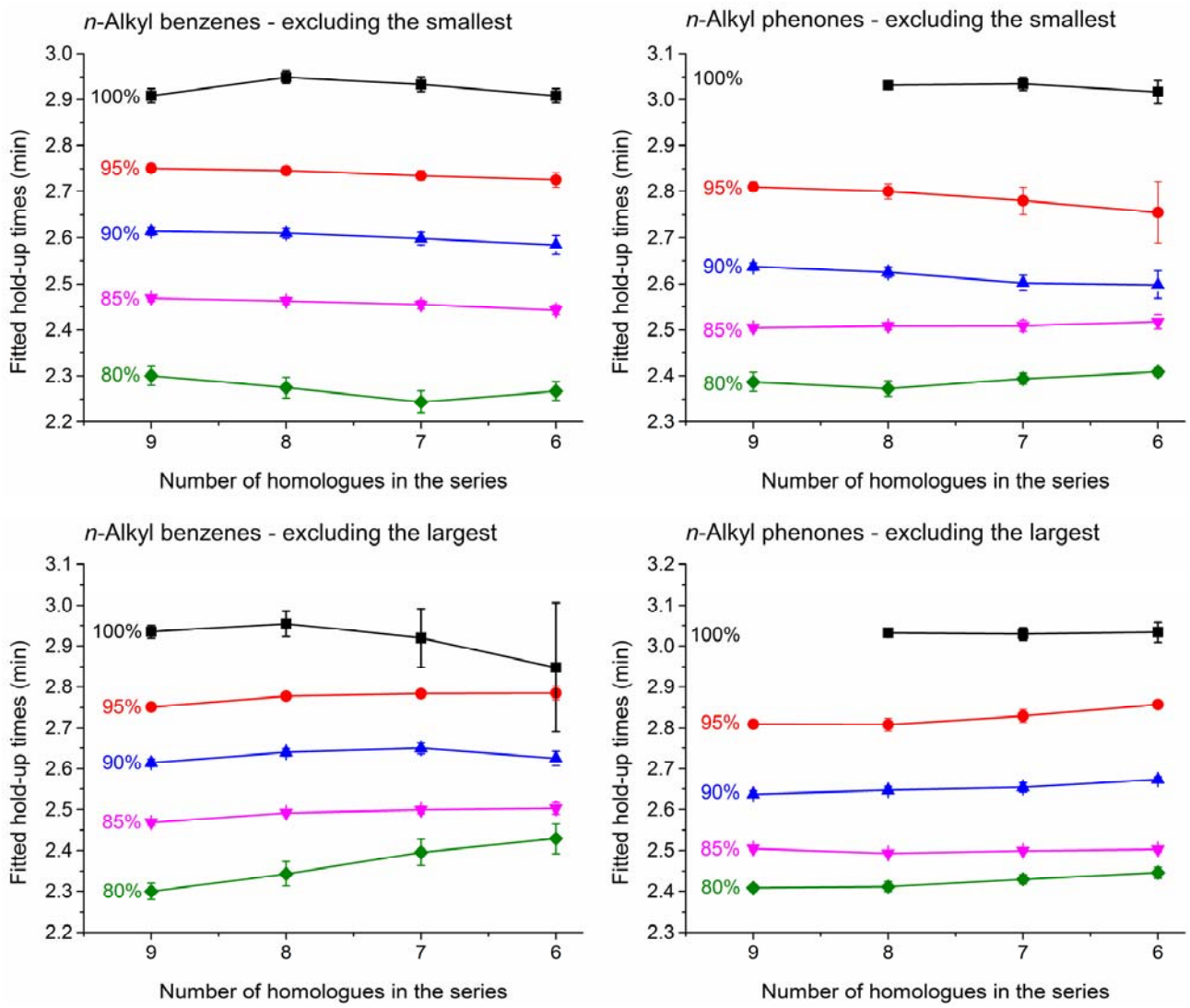

Figure 3. Effect of the number of homologues on fitted hold-up times (Eq. (7)) for *n*-alkyl benzene and *n*-alkyl phenone series in MP containing ACN. For each complete series (N=9) the number of homologues considered in the fittings were reduced excluding one (N=8), two (N=7), or three (N=6) of the largest or the smallest compounds (Table 1). Error bars show the standard error of the fitted hold-up time.

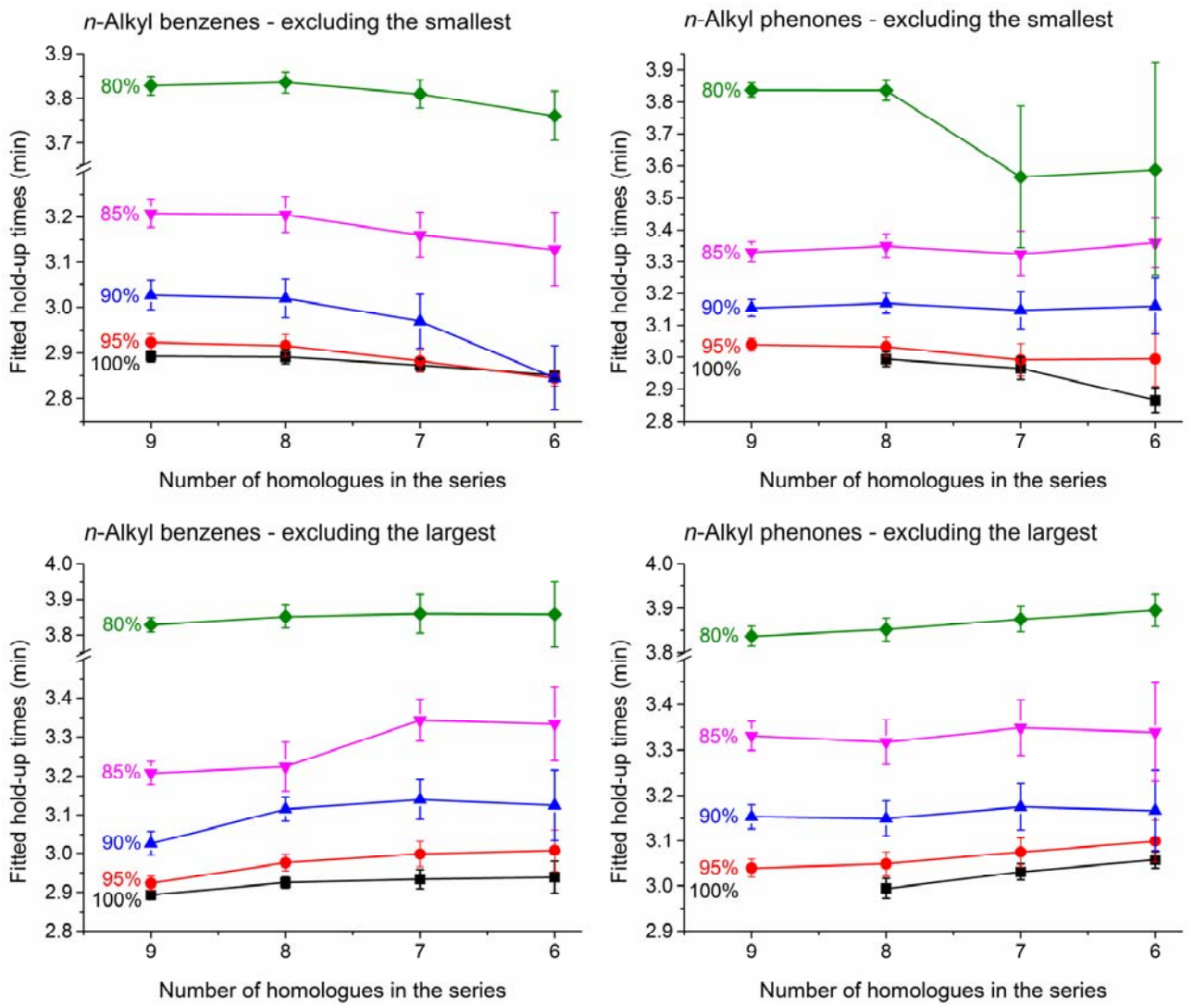

Figure 4. Effect of the number of homologues on fitted hold-up in MP containing MeOH.

REFERENCES

[1] G. Jin, Z. Guo, F. Zhang, X. Xue, Y. Jin, X. Liang, Study on the retention equation in hydrophilic interaction liquid chromatography, Talanta. 76 (2008) 522–527. doi:10.1016/j.talanta.2008.03.042.

[2] M.C. García-Alvarez-Coque, G. Ramis-Ramos, J.R. Torres-Lapasió, C. Ortiz-Bolsico, Modeling of retention in reversed phase liquid chromatography, Anal. Sep. Sci., Wiley-VCH Verlag GmbH & Co. KGaA, 2015. doi:10.1002/9783527678129.assep009.

[3] K.S. Yun, C. Zhu, J.F. Parchar, Theoretical relationships between the void volume, mobile phase volume, retention volume, adsorption, and Gibbs free energy in chromatographic processes, Anal. Chem. 67 (1995) 613–619. doi:10.1021/ac00099a020.

[4] X. Li, A.M. Hupp, V.L. McGuffin, The thermodynamic and kinetic basis of liquid chromatography, Adv. Chromatogr. (Boca Raton, FL, United States). 45 (2007) 1–88.

[5] K. Valko, Physicochemical and biomimetic properties in drug discovery, Wiley, Hoboken, NJ, United States, 2014.

[6] IUPAC. Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford (1997). XML on-line corrected version: http://goldbook.iupac.org (version 2.3.3, 2014-22-04). doi:10.1351/goldbook.

[7] C.F. Poole, S.K. Poole, Foundations of retention in partition chromatography, J. Chromatogr. A. 1216 (2009) 1530–1550. doi:10.1016/j.chroma.2008.10.092.

[8] G.E. Berendsen, P.J. Schoenmakers, L. de Galan, G. Vigh, Z. Varga-puchony, J. Inczédy, On the determination of the hold-up time in reversed phase liquid chromatography, J. Liq. Chromatogr. 3 (1980) 1669–1686. doi:10.1080/01483918008064759.

[9] C.A. Rimmer, C.R. Simmons, J.G. Dorsey, The measurement and meaning of void volumes in reversed-phase liquid chromatography, J. Chromatogr. A. 965 (2002) 219–232. doi:10.1016/S0021-9673(02)00730-6.

[10] D. V. McCalley, U.D. Neue, Estimation of the extent of the water-rich layer associated with the silica surface in hydrophilic interaction chromatography, J. Chromatogr. A. 1192 (2008) 225–229. doi:10.1016/j.chroma.2008.03.049.

[11] N.P. Dinh, T. Jonsson, K. Irgum, Probing the interaction mode in hydrophilic interaction chromatography, J. Chromatogr. A. 1218 (2011) 5880–5891. doi:10.1016/j.chroma.2011.06.037.

[12] N.P. Dinh, T. Jonsson, K. Irgum, Water uptake on polar stationary phases under conditions for hydrophilic interaction chromatography and its relation to solute retention, J. Chromatogr. A.

1320 (2013) 33–47. doi:10.1016/j.chroma.2013.09.061.

[13] M.H. Abraham, Scales of solute hydrogen-bonding: their construction and application to physicochemical and biochemical processes, Chem. Soc. Rev. 22 (1993) 73–83. doi:10.1039/cs9932200073.

[14] M.H. Abraham, J.C. McGowan, The use of characteristic volumes to measure cavity terms in reversed phase liquid chromatography, Chromatographia. 23 (1987) 243–246. doi:10.1007/BF02311772.

[15] ACD/Labs, Advanced Chemistry Development, Inc., Toronto, ON, Canada. www.acdlabs.com.

[16] N. Ulrich, S. Endo, T.N. Brown, N. Watanabe, G. Bronner, M.H. Abraham, K.U. Goss, UFZ-LSER database v 3.2 [Internet], Leipzig, Germany, Helmholtz Centre for Environmental Research-UFZ, (2017). http://www.ufz.de/lserd.

[17] W.K. Al-Thamir, J.H. Purnell, C.A. Wellington, R.J. Laub, Evaluation of dead space in chromatographic systems, J. Chromatogr. A. 173 (1979) 388–391. doi:10.1016/S0021-9673(00)92308-2.

[18] L. Didaoui, A. Touabet, A.Y. Badjah Hadj Ahmed, B.Y. Meklati, W. Engewald, Evaluation of dead time calculation in reversed-phase liquid chromatography using a multiparametric mathematical method, HRC J. High Resolut. Chromatogr. 22 (1999) 559–564. doi:10.1002/(SICI)1521-4168(19991001)22:10<559::AID-JHRC559>3.0.CO;2-R.

[19] M. Shibukawa, Y. Takazawa, K. Saitoh, Measurement of mobile-phase volume in reversed-phase liquid chromatography and evaluation of the composition of liquid layer formed by solvation of packing materials, Anal. Chem. 79 (2007) 6279–6286. doi:10.1021/ac0701839.

[20] M. Montes, J.L. Usero, A. Del Arco, C. Izquierdo, J. Casado, Free energy correlations: dead volume and the reversed-phase high-performance liquid chromatographic capacity factor in the interaction index model, J. Chromatogr. A. 481 (1989) 97–109. doi:10.1016/S0021-9673(01)96756-1.

[21] D.P. Nowotnik, R.K. Narra, A comparison of methods for the determination of dead time in a reversed-phase high-performance liquid chromatography system used for the measurement of lipophilicity, J. Liq. Chromatogr. 16 (1993) 3919–3932. doi:10.1080/10826079308019677.

[22] S. Pous-Torres, J.R. Torres-Lapasió, M.C. García-Álvarez-Coque, Performance of markers and the homologous series method for dead time estimation in reversed-phase liquid chromatography, J. Liq. Chromatogr. Relat. Technol. 32 (2009) 1065–1083. doi:10.1080/10826070902841372.

[23] R.J. Laub, S.J. Madden, Solute retention in column liquid chromatography. V. The column dead volume, J. Liq. Chromatogr. 8 (1985) 173–186. doi:10.1080/01483918508067070.

[24] E. Lázaro, C. Ràfols, M.H. Abraham, M. Rosés, Chromatographic estimation of drug disposition properties by means of immobilized artificial membranes (IAM) and C18 columns, J. Med. Chem. 49 (2006) 4861–4870. doi:10.1021/jm0602108.

[25] C. Reichardt, T. Welton, Solvents and solvent effects in organic chemistry, Wiley-VCH, Weinheim, 2011.

[26] E.D. Katz, C.H. Lochmüller, R.P.W. Scott, Methanol-water association and its effect on solute retention in liquid chromatography, Anal. Chem. 61 (1989) 349–355. doi:10.1021/ac00179a013.