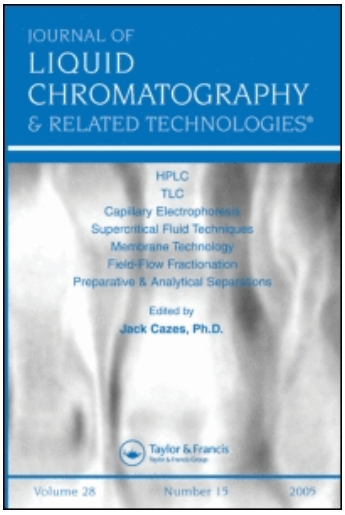

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Pous-Torres, S. , Torres-Lapasió, J. R. and García-Álvarez-Coque, M. C.(2009) 'Performance of Markers and the Homologous Series Method for Dead Time Estimation in Reversed-Phase Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 32: 8, 1065 — 1083
To link to this Article: DOI: 10.1080/10826070902841372
URL: http://dx.doi.org/10.1080/10826070902841372

Journal of Liquid Chromatography & Related Technologies®, 32: 1065–1083, 2009

ISSN: 1082-6076 print/1520-572X online
DOI: 10.1080/10826070902841372

Performance of Markers and the Homologous Series Method for Dead Time Estimation in Reversed-Phase Liquid Chromatography

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Abstract: Two methods for dead time estimation (the use of markers and the homologous series mathematical method) are revised. Out of twelve assayed common markers, only KBr, KI, tartrazine, thiourea, uracil, and urea yielded retention times independent of the mobile phase composition in the range 10–90% acetonitrile, using a Zorbax Eclipse XDB−C18 column. On the other hand, the quality of the estimations provided by the homologous series method was limited by the mathematical approach and the data quality. With this method, the estimated dead time is an extrapolated value, which is severely affected by the data of the most retained compounds that act as leverage points, biasing the result. The sequential elimination of the most retained compounds attenuates this problem. This means that the homologous series should contain at least four compounds with low retention. Otherwise, overestimations of dead time are yielded.

Keywords: Convergence method, Dead time, Homologous series, Markers, Nitrosamines, Reversed-phase liquid chromatography

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INTRODUCTION

The void volume in liquid chromatographic systems has been defined as "the volume of mobile phase that fills the space between the injector and the detector cell, which includes the accessible interstitial volume (the volume between column particles) and the intraparticulate volume (the pores of the column packing), as well as the volume of tubing and any other component in the system".[1,2] Related with this concept is the dead time, which is the time that an ideal unretained compound (i.e., a compound that does not interact with the stationary phase) needs to cross the distance between the injector and the detector when eluted at constant flow rate.[3,4]

Dead time estimation in chromatographic systems is the basis for the calculation of retention factors:

$$k = \frac{t_R - t_0}{t_0} \quad (1)$$

where t_R is the solute retention time and t_0 the dead time. Usually, the gross dead time (t_0^g, the sum of the column dead time and the extra column contributions) is taken as dead time.[5] Corrected retention times can be calculated using either the gross values or the values obtained once subtracted from the extra column contributions: $t_R^g - t_0^g = (t_R + t_{extra}) - (t_0 + t_{extra}) = t_R - t_0$. However, strictly, in order to calculate k values, the corrected retention times should be divided by the column dead time and not by the gross dead time. Accurate knowledge of retention factors is needed in the prediction of retention and resolution for the optimisation of chromatographic separation, in the determination of partition coefficients and other thermodynamic parameters, and in the establishment of correlations with several properties.

The definition of dead time involves two main difficulties, especially for reversed-phase liquid chromatography (RPLC):[6]

(i) The molecules of organic solvent are adsorbed onto the stationary phase, forming a layer and thus reducing the accessible volume between injector and detector. The situation becomes more complex owing to the fact that the stronger components of the mobile phase are preferentially associated to the stationary phase.
(ii) Solute molecules may be partially or completely excluded from the stationary phase pores. This means that different solutes will have different dead times associated, since small solutes and portions of some larger solutes can access the average pores, while other solutes may not be able to enter the pores. To this effect, the electrostatic exclusion should be added.

In RPLC, an unambiguous definition of mobile phase and stationary phase volumes, or the boundary between both phases, is not easy.[6,7] The results yielded by different methods reported for dead time estimation can be expected to differ because they measure different properties.[8] The discussion and controversy on this topic has been kept alive during decades, giving rise to several review reports.[1,3,5,6,8–11] The general opinion is that there is no generally accepted method for the accurate evaluation of dead time yet.

The methods that have been reported for dead time estimation in RPLC have been classified as static and dynamic. In the former case, there is no flow and the column is kept at atmospheric pressure. In the latter, the mobile phase is flowing, and there is a linear pressure gradient along the column. The most usual static method is the pycnometry or weight difference method. This consists in filling the packed column successively with two solvents of sufficiently different density (e.g., carbon tetrachloride and methanol) and weighting.[8,12] The total volume is then obtained from the differences in density and weight. In another method, the column is flushed with water and dried with a nitrogen stream.[12,13] Both methods give direct information about the column void volume, but they ignore the solvation of the stationary phase by the components of the mobile phase. Thus, unless a correction is made for the solvation layer, they provide an overestimation of the true column dead time. They also involve an appreciable degree of error derived from the estimation of a small magnitude from the subtraction of two larger magnitudes (i.e., the weight of the column filled with each solvent, or filled with a solvent and dried). The one solvent method is impractical because, once the column is dried, it is often irreversibly damaged. A different static method consists of measuring the difference between the volumes of the empty column tube and the material packed inside the column. The implementation of this method requires the use of specific instruments and a significant amount of time, making it impractical for routine use.[8] Consequently, static methods are not commonly used.

Dynamic methods include the elution of unretained compounds (direct methods), such as the mobile phase components (in the so called ''minor disturbance method''), or markers, and the elution of homologous series (in the so called ''mathematical method''), which is an indirect method. The main problem of direct methods is that all test compounds are either slightly retained or excluded from the stationary phase.[5] Also, the measurements depend on the experimental conditions.[8]

A solvent disturbance or system peak will be obtained by injecting a mobile phase component (water or organic solvent), or a volume of mobile phase with a composition slightly different from that currently used.[6] In general, a refractive index (RI) detector is not necessary to measure the solvent disturbance peak; frequently, UV detectors evidence

changes in RI at low wavelengths. The most weakly bound mobile phase component (i.e., water in RPLC) has been proposed as the best to be injected, as it should not be a part of the solvation layer. However, when pure water is injected, at least two peaks are observed: the peak of pure water, and a peak produced by the slight excess of organic solvent in the mobile phase immediately following the water, due to the disturbance of the equilibria between mobile phase and stationary phase (i.e., water vacancy peak).

The disturbance peak will be undetected if the injected solvent has exactly the same composition as the mobile phase. For this reason, isotopically labelled mobile phase components are used (deuterated compounds, usually $^{2}H_2O$, or radioactive compounds such as $^{3}H_2O$ or $^{14}CH_3CN$).[1] The great disadvantage of these isotopic compounds is the detection. Deuterated compounds are also UV transparent, and an RI detector may be needed. In addition, vacancy peaks complicate the detection, and the sensitivity can be low for certain hydro-organic mixtures. A scintillation counter has been used to detect radioactively labelled components.[3] These components are far less common than the deuterated ones, due to problems with the solvent disposal, as well as the possible contamination of column and injector (among others).

The injection of ideally UV-absorbing markers, either organic (e.g., acetone, benzoic acid, *N,N*-dimethylformamide, nitrobenzene, picric acid, or uracil), or inorganic (e.g., KI and KNO_3, NaCl, $NaNO_3$, and $NaNO_2$), is a common practice to determine the dead time due to its practical simplicity. In this case, a possible retention of the injected compound is ignored. This has been claimed to be, however, the most suspicious of the methods.[6] The ideal unretained compound should be small enough to access the whole available stationary volume, and hydrophilic enough to stay out of the stationary phase. This is not easy to find, since different small neutral molecules show retention times that decrease at increasing mobile phase hydrophobicity.

The inorganic salt method is the most arguable. Dead time measured with inorganic salts is affected by the mobile phase composition, pH, ionic strength, ionic volume, and amount of the injected marker. This has been interpreted as the exclusion of charged species from the pores of the packing material (Donnan effect), due to the presence of residual silanol groups. Consequently, the dead time measured using inorganic salts can vary between the total volume of the mobile phase in the column and a value close to the interparticle volume, depending on the experimental conditions.[3,5] Addition of a buffer solution or a large concentration of salts (even of the marker) mask the effect of the charged silanols, and makes the marker ions able to penetrate the pores. A final problem with inorganic salts is their low solubility in modifier rich eluents. In spite of these observations, inorganic salt markers have been recommended to

study the chromatographic behaviour of ionisable compounds, arguing that they match more closely.[14]

Dead time estimation from the retention of successive members in a homologous series obviates the difficulties associated to the selection of truly unretained compounds. The approach has been suggested to be theoretically ideal.[6] It is based on the Martin's rule,[15] the linear relationship between the logarithm of retention factor and the homologue carbon number, and calculates the dead time by extrapolation. It has, however, several drawbacks: it is time consuming, data processing (non-linear regression) is needed, it requires highly precise and accurate data, and the assumption of linearity throughout the whole series does not seem to be always valid in RPLC; deviations from linearity have been observed for the smaller homologues and for the homologues exceeding the alkyl chain of the bonded phase. A minimum of three homologues is required in order to fit the mathematical model, but at least four are recommended. The choice of the homologous series is based on the availability, solubility in the mobile phase, retention, and detection. A single series would not cover the entire range of mobile phase compositions, since the solubility and retention of the highest homologues become rapidly inappropriate with decreasing organic solvent content.[3,6,9,16]

In an early work, Berendson et al.[9] claimed that the results obtained from this approach are (or should be) independent of the homologous series. However, most authors have found a significant dependence. Thus, Krstulovic et al.[16] observed a considerable variation in dead time values for different homologous series, especially when a limited number of homologues with predominantly low carbon number were used. Nowotnik and Narra[17] obtained reasonably consistent dead time values derived from the alkanol series, whereas the values from alkylbenzenes differed significantly. Other authors[2,18] indicated that dead time estimation from the retention data of alkylbenzenes can be unreliable.

Although not sufficiently discussed, some insight in the reason for the different dead time estimations using different homologous series is found in the literature. Thus, Haken et al.[19] pointed out that mathematical dead time calculations are strongly influenced by small changes in retention times of the compounds in the homologous series. Krstulovic et al.[16] observed a considerable scattering of dead time values calculated from the mathematical approach when applied to various homologous series, and interpreted the results as due to the imprecision of retention data for insufficiently retained homologues, and/or the existence of curvature for the low homologues. Also, contrary to some other findings which showed that with reasonable cautions, good estimates of dead time could be obtained even for a few homologues, the authors found a critical dependence of the dead time estimation on both the number and choice of homologues, at least in some cases. According to Laub and Madden,[20]

dead time is affected substantially by the choice of the homologues set. Finally, Didaoui et al.[21] found that alkyl aryl ketones showed significantly lower values of dead time compared to those obtained for alka–2–ones and 1–nitroalkanes, and associated the difference to the appreciably higher retention of the former, and the fact that the dead time is obtained by extrapolation.

In this work, the adequacy of several common markers for dead time estimation and the conditions of application of the homologous series method are checked.

EXPERIMENTAL

Compounds and Chromatographic Conditions

Several common dead time markers: acetone (Scharlau, Barcelona, Spain), benzoic acid (Probus, Barcelona), *N,N*-dimethylformamide, picric acid, tartrazine, and urea (Panreac, Barcelona), floroglucinol dihydrate, 2–nitrobenzoic acid, and uracil (Acros Organics, Geel, Belgium), KBr (Prolabo, Fontenay-sous-bois, France), KI (Guinama, Valencia, Spain), and thiourea (Baker, Phillipsburg, NJ, USA), were examined. Stock solutions were prepared by dissolving the compounds in a few milliliters of water with the aid of an ultrasonic bath, and diluting them with an acetonitrile-water mixture. The injected solutions (10 μg/mL, except for KBr which was 200 μg/mL) were prepared in a volume of mobile phase. The markers were eluted through a Zorbax Eclipse XDB−C18 column (150 × 4.6 mm, 5 μm particle size, Agilent, Waldbronn, Germany), using acetonitrile (Scharlau) in the 0−100% range, buffered at pH 3 with citric acid monohydrate (Panreac) and NaOH (Scharlau). Compound solutions and mobile phases were filtered through 0.45 μm nylon membranes (Cameo and Magna, respectively, Osmonics, Herental, Belgium). Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

The mathematical method was applied to the nitrosamine and alkylbenzene homologous series. The former consisted of (number of carbons in the series) *N*-nitrosodimethylamine (C2), *N*-nitrosomethylethylamine (C3), *N*-nitrosomethyl-*n*-propylamine (C4), *N*-nitrosomethyl-*n*-butylamine (C5), *N*-nitrosodi-*n*-propylamine (C6), *N*-nitroso-*n*-propyl-*n*-butylamine (C7), and *N*-nitrosodi-*n*-butylamine (C8). These compounds were eluted from a Phase Separation Spherisorb S5CN column (100 × 0.46 mm, 5 μm particle size), using acetonitrile and methanol in the ranges 20−55% and 30−60% (v/v), respectively.[22]

The alkylbenzene homologous series consisted of toluene (Scharlau), ethylbenzene, and butylbenzene (Aldrich, St. Louis, MO, USA),

propylbenzene (Acros Organics), and pentylbenzene (Fluka, Buchs, Switzerland). The same column used for the markers was used, and the elution was carried out with unbuffered acetonitrile-water mixtures in the 0–100% range.

Apparatus and Data Processing

The experimental series were carried out with a chromatographic system (Agilent, Waldbronn, Germany), equipped with an isocratic pump (Series 1200), an automatic sampler, a thermostated column module, and a variable wavelength detector (Series 1100), all governed by an Agilent HPChemStation B.02.01.

In all instances, the flow rate was set at 1 mL min^{-1}, the injected volume was 5 μL, and the temperature was kept constant at 25°C. Triplicate injections were made. Signals were monitored at 254 nm, with the exception of KBr and urea (207 nm).

Data treatment was carried out with software developed in Visual Basic 6.0 (Microsoft Corporation, Seattle, WA, USA).

THEORY

Correlations Between Log *k* and Hydrophobicity

A large number of reports informing about the relationships between log k and a number of properties correlated with hydrophobicity can be found in the literature. The most usual are the following:

$$\log k = c_{0,1} + c_{1,1} n_C \tag{2}$$

$$\log k = c_{0,2} + c_{1,2} \log P_{o/w} \tag{3}$$

$$\log k = c_{0,3} + c_{1,3} \varphi \tag{4}$$

where n_C is the number of carbons in the homologue, $P_{o/w}$ the octanol-water partition coefficient, and φ (as in (4)) mobile phase. It should be noted that the linearity is only strictly valid in relatively narrow ranges of these parameters. In Equations (2) and (3), the chromatographic environment is kept constant, and the data involved in the fittings correspond to solutes of diverse hydrophobicity. The first equation is restricted to homologous series, whereas the second one can be applied to any compound in the absence of specific contributions to retention (e.g., ionic interactions with silanol groups or steric effects). In contrast, Equation (4) is based on the alteration of the chromatographic environment (i.e., mobile phase composition), attending to the retention behaviour of a

single compound. It can be applied to any kind of solute, provided that eventual secondary equilibria, such as protolysis, are not shifted along the series.

Homologous Series Mathematical Method

As indicated in the introduction section, Equation (2) has been the basis of the "mathematical method" for dead time estimation. This method relies on an extrapolation to get the dead time, and is based on the combination of Equations (1) and (2):

$$t_R = t_0 (1 + k) = t_0 (1 + k_0 e^{c_{1,1} n_C}) \quad (5)$$

where k_0 is the residual retention factor for $n_C = 0$, and $c_{1,1}$ the slope of the log k versus n_C relationship (Equation (2)). The parameters t_0, k_0, and $c_{1,1}$ can be calculated by non-linear regression. It was checked that Equation (5) written as:

$$t_R = t_0 + e^{a_1 + b_1 n_C} \quad (6)$$

where a_1 and b_1 are fitting parameters, shows more favourable convergence properties. However, these parameters are collinear with t_0, and consequently, they are more uncertain than the equivalent parameters in Equation (5). In spite of this, the estimation of t_0 may be sufficiently precise and unbiased. The fittings were performed by applying the Powell's method,[23] and checked to be fast and accurate enough in all instances.

Retention versus Composition Relationships

A new approach was developed, using internal models relating log k and the solvent content, such as Equation (4). This approach is implicitly accepting that the dead time is not affected by changes in mobile phase composition, a hypothesis that has been suggested by some authors.[21,24] Using the linear dependence outlined in Equation (4), the following is obtained:

$$t_R = t_0 (1 + k) = t_0 (1 + k_0 e^{c_{1,3} \varphi}) \quad (7)$$

$$t_R = t_0 + e^{a_3 + b_3 \varphi} \quad (8)$$

The latter equation offers, again, better convergence properties.

RESULTS AND DISCUSSION

Dead Time Estimation Using Common Markers

The retention of 12 compounds (organic and inorganic), which are often used as dead time markers, was measured at several mobile phase compositions between 10 and 90% acetonitrile. Figure 1 shows chromatograms for 10, 50, and 90% acetonitrile. When considering the whole composition range, the change in retention time was remarkable for 2–nitrobenzoic, benzoic, and picric acids (from 7.52 to 1.46, 33.7 to 1.53, and 47.4 to 1.39 min, respectively). A minor change was observed for dimethylformamide, acetone, and floroglucinol (from 1.99 to 1.49, 2.48 to 1.54, and 2.33 to 1.36 min), being the change non-significant for KBr, KI, tartrazine, thiourea, uracil, and urea. Dead time estimations for the latter six markers, obtained by direct measurement of the time at the peak maximum, were (min): 1.23 ± 0.06, 1.33 ± 0.07, 1.22 ± 0.05, 1.38 ± 0.08, 1.36 ± 0.10, and 1.22 ± 0.06, respectively, with a global mean of 1.29 ± 0.07. The measurement of the disturbance peak for several chromatograms obtained with the same column in the 50–90% acetonitrile range gave the following estimation: 1.21 ± 0.05 min.

Repeatability of retention time measurements was obtained by a ten fold injection of acetone (1.6812 ± 0.0003), tartrazine (1.1599 ± 0.0011), and uracil (1.2793 ± 0.0002), using a mobile phase of 50% acetonitrile. The extra column dead time was measured by triplicate injection of four compounds (acetone, *N,N*-dimethylformamide, tartrazine, and uracil) in the chromatographic system without the column, being $t_{ext} = 0.0912 \pm 0.0014$ min.

Homologous Series Mathematical Method

In the most recent review on dead time estimation,[6] the authors expressed that "the dead time calculated according to the homologous series mathematical approach should be comparable to one another, no matter which homologous series and how many homologues are used". Common sense indicates that this method should yield the same value of dead time, independently of the homologues nature, provided exclusion effects are similar. In our opinion, the different results obtained with different homologous series rely mostly on the mathematical approach and the data quality, since the dead time is an extrapolated value. The problem arises from the large weight in the fitting given to the most retained compounds, whose data act as leverage points. The bias increases for series where the homologues show large retention (i.e., highly hydrophobic compounds or mobile phases of low elution

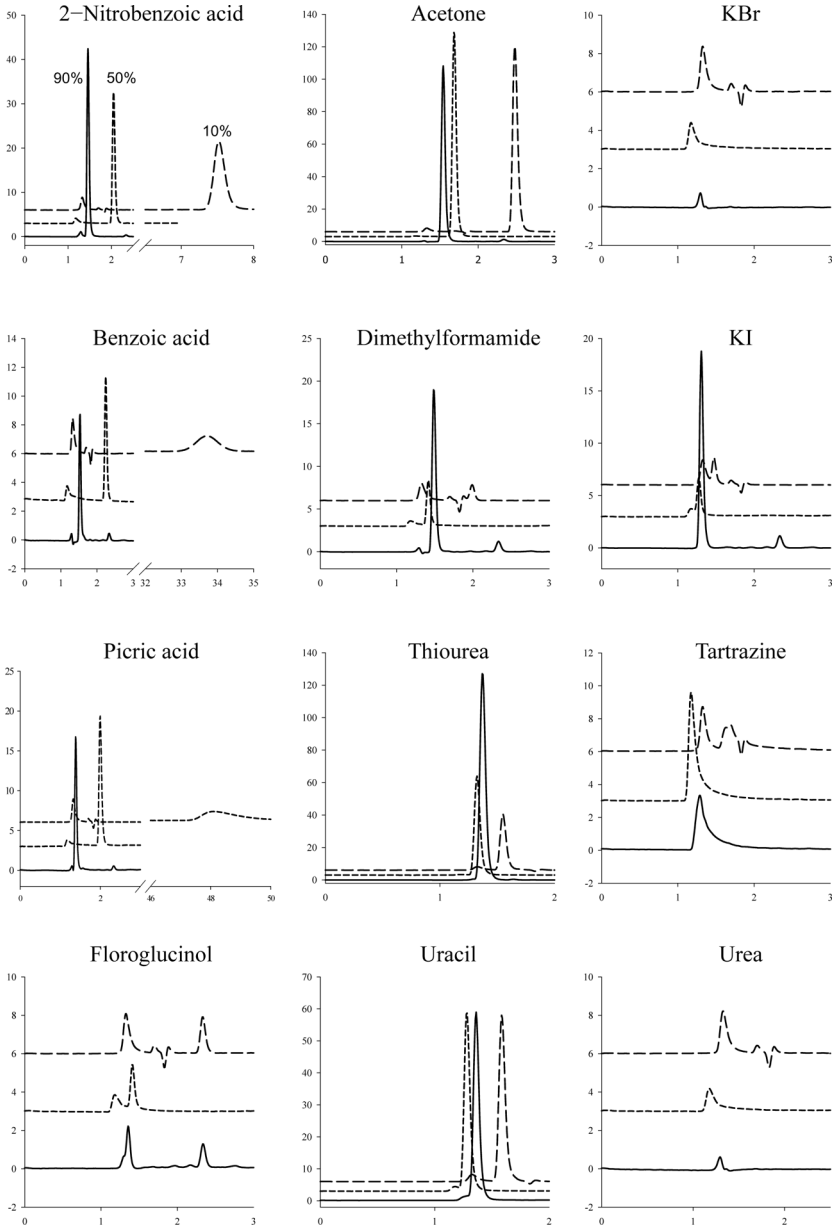

Figure 1. Chromatograms for some usual dead time markers at three mobile phase compositions.

strength). Nevertheless, the deviation from linearity reported for some low homologues should not be discarded.

Krstulovic et al.[16] developed a convergence test, which consisted of calculating the dead time values for all available homologues in a series, as well as for groups of homologues obtained by sequential elimination of either the lowest or the highest in the series. In certain cases, dead time estimations changed significantly with a change in the number of homologues included, evidencing that the choice of homologues is of critical importance for obtaining consistent results. Also, the authors found that the estimations made after eliminating some homologues from several series (alkanes, alkylbenzenes, methyl esters, chloroalkanes, and alcohols) were rather similar, and in close agreement with those obtained by injection of ^2H_2O. Nowotnik and Narra[17] applied the convergence test to eight homologues of the *n*-alkanol and *n*-alkylbenzene series, and indicated that the method was unreliable with a number of points below four.

A different approach was suggested by Montes et al.,[22] who used weighting factors over the dependent variable (i.e., the retention, which is the source of error). The weights were inversely related to the difference between the retention time and the estimated dead time. In fact, the elimination of homologues within a series is equivalent to applying a zero weighting factor. It should be noted that the selection of the weighting factor is arbitrary and affects the results.

We applied the convergence approach to two homologous series (nitrosamines and alkylbenzenes), but only the highest homologues were sequentially eliminated. The data corresponds to seven nitrosamines with $n_C = 2-8$, eluted with eight acetonitrile-water mixtures in the range 20–55%, and seven methanol-water mixtures in the range 30–60%.[22] Nitrosamines include two hydrocarbon chains. However, the contribution of the carbon chain to retention is independent of its position. The data for the second series corresponds to five alkylbenzenes with $n_C = 1-5$, eluted with acetonitrile-water in the range 50–100%.

Table 1 shows the estimated dead time from Equation (6) for nitrosamines eluted with acetonitrile and methanol at diverse mobile phase composition, using the data of the whole series and after eliminating one by one the highest homologues, up to keeping four ($n_C = 2$–5). The retention time of the highest processed homologue is given in each case. As observed, data elimination yields variations in the extrapolated value of t_0, but this tended to stabilize when the maximal retention time ($t_{R,max}$, retention time for the highest homologue) was close to 5–6 min. We adopted the arbitrary criterion of averaging the estimations obtained for $t_{R,max}$ of approximately twice the estimated t_0.

According to this criterion (see bold values in Table 1), the mean t_0 value was 2.62 ± 0.13 min for acetonitrile, and 2.84 ± 0.09 min for

Table 1. Estimated dead times (min) from Equation (6) for a series of nitrosamines, along the sequential elimination of homologues[a]

	Mobile phase composition (%, v/v)							
Acetonitrile	20	25	30	35	40	45	50	55
$n_C = 2$–8	3.12 (12.76)	2.99 (9.44)	2.83 (7.23)	2.71 (6.06)	**2.60** (5.09)	**2.56** (4.59)	**2.46** (4.05)	**2.60** (3.67)
$n_C = 2$–7	2.94 (8.61)	2.84 (7.00)	2.73 (5.85)	**2.62** (5.17)	**2.49** (4.55)	**2.60** (4.24)	**2.56** (3.85)	**2.62** (3.67)
$n_C = 2$–6	2.79 (6.44)	2.73 (5.62)	**2.62** (4.96)	**2.61** (4.57)	**2.49** (4.16)	**2.54** (3.93)	**2.43** (3.65)	**2.66** (3.42)
$n_C = 2$–5	**2.93** (5.14)	**2.87** (4.71)	**2.80** (4.35)	**2.81** (4.11)	**2.66** (3.85)	**2.59** (3.69)	**2.41** (3.49)	**2.64** (3.32)
Methanol	30	35	40	45	50	55	60	
$n_C = 2$–8	3.16 (19.71)	3.08 (12.27)	3.01 (8.67)	2.97 (6.76)	**2.97** (5.45)	**2.90** (4.47)	**2.88** (3.89)	
$n_C = 2$–7	3.00 (12.33)	2.97 (8.60)	2.91 (6.65)	**2.88** (5.56)	**2.84** (4.73)	**2.84** (4.11)	**2.82** (3.71)	
$n_C = 2$–6	2.83 (8.46)	2.77 (6.51)	**2.78** (5.42)	**2.75** (4.79)	**2.77** (4.29)	**2.81** (3.85)	**2.66** (3.56)	
$n_C = 2$–5	3.02 (6.24)	**2.99** (5.24)	**2.93** (4.63)	**2.91** (4.26)	**2.83** (3.93)	**2.86** (3.65)	**2.66** (3.45)	

[a]The retention time of the highest homologue ($t_{R,max}$ in min) is given in parenthesis. The estimated dead times for $t_{R,max} \leq 2t_0$ are marked in bold.

methanol. When the data for $n_C = 2-5$ (where only four homologous are included) was not considered, $t_0 = 2.56 \pm 0.06$ and 2.82 ± 0.08, respectively. The estimations made by Montes et al. using the same data and applying weighting factors[22] were: 2.68 ± 0.15 and 2.90 ± 0.04, respectively (mean estimations of the dead time values at different mobile phase compositions).

The retention times of alkylbenzenes were appreciably larger than for nitrosamines, with $t_{R,max} = 46.7$ min for the homologue with $n_C = 4$ eluted with 50% acetonitrile. As the series contained only five homologues, only one homologue could be eliminated upon application of the convergence method. Further eliminations gave inconsistent estimations owing to the lack of information. The estimated t_0 was 1.58 ± 0.08 min, value which should be compared with the estimation made with the markers: 1.29 ± 0.07 min.

Figure 2 depicts the correlations of log k versus carbon number, and log k versus organic solvent content, for nitrosamines eluted with acetonitrile and methanol, and for alkylbenzenes eluted with acetonitrile, where t_0 was taken as 2.62, 2.84, and 1.29 min, respectively. In general, good linearity is observed, with small deviations in some cases for the weakest mobile phases or slowest solutes.

Retention versus Composition Relationships

In the previous section, the data in a matrix where the homologue number correspond to the rows, and the mobile phase composition to the columns, were treated row-wise (Equation (6)). We show here a similar treatment to the homologous series method, processing the data column-wise (Equation (8)).

Only a few reports on dead time estimation include studies on the effect of mobile phase composition. However, there is a belief that dead time changes with the organic solvent contents. In fact, this is not surprising in view of the results shown above with several common markers, which exhibited significant and inconsistent changes in retention upon decreasing the solvent elution strength. Knox and Kaliszan estimated the total volume of eluent components within the column bed after injection of isotopically labelled samples of all eluent components.[24] They found that the dead time did not depend on mobile phase composition, with highly similar values for different solvent mixtures over a wide composition range. Also, Wainwright et al.[18] reported a negligible variation of the estimated dead times with the organic modifier content for several homologous series (alkan−2−ones, alkyl aryl ketones, and 1–nitroalkanes), with similar values for acetonitrile and methanol.

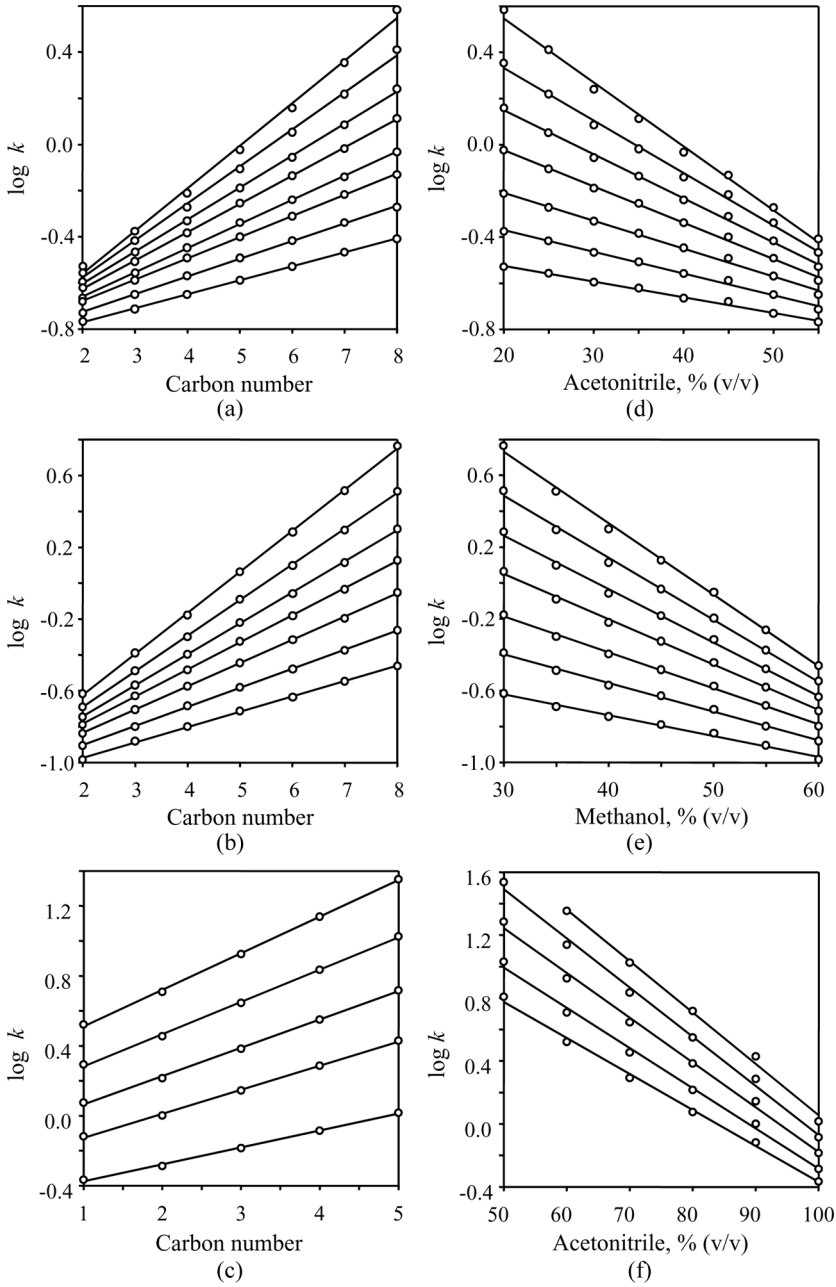

Figure 2. Correlation between log k and homologue carbon number (a,b,c), or organic solvent content (d,e,f) for nitrosamines (a,b,d,e) and alkylbenzenes (c,f), obtained using the estimated dead times. From bottom to top: (a,b) $n_C = 2$ to 8, (c) $n_C = 1-5$, (d) 20, 25, 30, 35, 40, 45, 50, and 55% acetonitrile, (e) 30, 35, 40, 45, 50, 55, and 60% methanol, (f) 50, 60, 70, 80, 90, and 100% acetonitrile.

Table 2. Estimated dead times (min) from Equation (8) for individual homologues[a]

Composition range (%, v/v)	Number of carbon atoms (n_C) 1	2	3	4	5	6	7	8
Nitrosamines and acetonitrile-water								
20–55		2.19 ± 0.69 (3.42)	2.16 ± 0.41 (3.75)	2.32 ± 0.25 (4.27)	2.60 ± 0.14 (5.14)	2.83 ± 0.13 (6.32)	3.08 ± 0.13 (8.61)	3.31 ± 0.13 (12.76)
25–55		2.19 ± 0.69 (3.37)	1.95 ± 0.56 (3.65)	1.90 ± 0.36 (4.06)	2.39 ± 0.15 (4.71)	2.67 ± 0.13 (5.57)	2.91 ± 0.13 (7.00)	3.16 ± 0.13 (9.44)
Nitrosamines and methanol-water								
30–60		2.87 ± 0.19 (3.59)	2.97 ± 0.11 (4.07)	3.00 ± 0.11 (4.85)	3.10 ± 0.12 (6.24)	3.24 ± 0.16 (8.27)	3.39 ± 0.09 (12.33)	3.64 ± 0.12 (19.71)
35–60		—[b]	2.59 ± 0.16 (3.83)	2.64 ± 0.07 (4.35)	2.76 ± 0.03 (5.24)	2.83 ± 0.12 (6.40)	2.95 ± 0.05 (8.60)	3.03 ± 0.14 (12.27)
Alkylbenzenes and acetonitrile-water								
50–100	1.73 ± 0.09 (9.81)	1.90 ± 0.13 (15.51)	2.20 ± 0.24 (26.79)	2.67 ± 0.42 (46.73)	—[c]			
60–100	1.41 ± 0.02 (5.70)	1.41 ± 0.05 (8.06)	1.41 ± 0.11 (12.41)	1.42 ± 0.07 (19.48)	1.43 ± 0.15 (31.10)			

[a]The retention time (min) for the weakest mobile phase is given in parenthesis.
[b]No appropriate convergence.
[c]The retention time of pentylbenzene was >65 min.

Assuming that the dead time is negligibly affected by the organic solvent content, we performed a straightforward fitting of Equation (8), carried out individually for each nitrosamine. We found, however, that the dead time depended strongly on the particular compound (Table 2). The dependence was even larger than that observed by applying the homologous series method at several mobile phase compositions (Table 1).

These results illustrate why Equation (8) or another similar was not applied before for dead time estimation. However, the observed behaviour should be again interpreted, at least partially, as due to the diverse range of retention for each solute and the insufficient accuracy in the measurement of retention times (and organic solvent content). Note that n_C is a scalar number, and the uncertainties in the fitting of Equation (6) should only be assigned to k. The mean dead time (considering only the solutes with a maximal retention time $t_{R,max} \leq 2t_0$) was 2.32 ± 0.20 and 2.11 ± 0.23 for nitrosamines eluted with acetonitrile in the ranges 20–55% and 25–55%, respectively, and 2.95 ± 0.07 and 2.66 ± 0.09 for nitrosamines eluted with methanol in the ranges 30–60% and 35–60%, respectively (further elimination of the weakest mobile phases yielded inconsistent results). These values should be compared with those obtained with the homologous series method: 2.62 ± 0.13 min for acetonitrile, and 2.84 ± 0.09 min for methanol.

The results did not improve significantly by changing the subjacent linear log k versus φ relationship (Equation (3)), using instead other alternative relationships (linear or quadratic with φ or the polarity parameter P_m^N).[25]

CONCLUSIONS

The retention of twelve common markers in the 10–90% acetonitrile range showed that only KBr, KI, tartrazine, thiourea, uracil, and urea are truly unretained, although with small differences, which should be attributed to their different accessibility to column pores and the existence of residual interactions. The selection of a unique marker is risky and requires checking that changes in mobile phase composition do not affect the dead time estimation.

The dead estimation derived from the homologous series approach depended on the mobile phase composition. This was explained as a consequence of the different retention time ranges included in the fittings. The dead time is an extrapolated value, severely affected by the data of the most retained compounds, which should be eliminated from the series. The sequential elimination of the highest homologous was checked to converge towards consistent dead time estimations. However, this

strategy is limited by the nature of the selected homologous series. Ideally, it should include at least four compounds with low retention. From the two assayed series, only nitrosamines were appropriate.

The use of retention versus composition relationships undergoes a similar problem; the estimation is more or less biased depending on the magnitude of the retention. In some cases, due to the narrow variability of retention times with mobile phase composition or the inaccuracy in the retention times or organic solvent contents, the individual fittings of Equation (3) yield strong biases. These results suggest that the simultaneous treatment of all available information (the data from several homologues and mobile phase compositions) could be beneficial.

ACKNOWLEDGMENTS

This work was supported by Project CTQ2007−61828/BQU (Ministerio de Educación y Ciencia of Spain, M.E.C) and FEDER funds. S.P.T thanks a FPI grant from the M.E.C.

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Received November 8, 2008
Accepted December 12, 2008
Manuscript 6441