Journal of Chromatography, 481 (1989) 97–109
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 753

FREE ENERGY CORRELATIONS: DEAD VOLUME AND THE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CAPACITY FACTOR IN THE INTERACTION INDEX MODEL

A DISCUSSION AND APPLICATION TO A NITROSAMINE SERIES

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(First received December 23rd, 1988; revised manuscript received July 5th, 1989)

SUMMARY

On the basis of a free energy correlation between distribution coefficients and the number of carbon atoms found in a series of nine nitrosamines, the column dead volume was determined for different column–mobile phase systems, using methanol and acetonitrile as the organic components of the mobile phase. Application of the interaction index model for reversed-phase liquid chromatography yielded a quadratic equation as the expression of the selectivity, α, without it being possible to dispense with the second degree term. It is shown that the variation in the capacity factor, k', with the composition of the organic component cannot be dealt with simplistically, since the relationship between the volumes of the stationary and mobile phases, $\varphi(x)$, varies considerably with composition. The existence of a convergence zone in plots of $\ln k'$ against the number of carbon atoms in the solute chain should not be interpreted as a characteristic property of the series, and may result from a compensation effect of the terms governing the dependence of the capacity factor on the eluent composition.

INTRODUCTION

The investigation of precise correlations between chromatographic parameters and experimental distribution coefficients is proving to be a useful approach to a better knowledge of the hydrophilic–lipophilic behaviour of many chemical species of biological interest[1].

The chemistry of N-nitrosamines has received increasing attention owing to the proven carcinogenic[2,3], mutagenic[4] and teratogenic[5] properties of such substances towards many animal species, including the primates. A report prepared for the Surgeon General of the United States stated that the majority of human cancers are caused by avoidable exposure to carcinogens[6] and recently the Congress of the United States itself published a background paper on the identification and regulation of carcinogens[7].

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Within the framework of research into the mechanisms of the formation and degradation of N-nitroso compounds, *cf.*, refs. 8–12, chromatography, among other techniques[13], has been used for the analysis and monitoring of such species[14].

The scope of this paper lies not so much in the use of chromatography as an analytical tool but rather in its possibilities for the study of the behaviour of N-nitroso compounds in hydrophilic–lipophilic media. Accordingly, results are reported on the correlations between the structures of N-nitrosamines (in terms of their numbers of carbon atoms) and their distribution coefficients in hydrophilic–lipophilic media on the one hand and their capacity factors on the other, and application of the interaction index model to the study of the reversed-phase behaviour of the same N-nitrosamine series.

PROBLEM

Eqn. 1 establishes the relationship between the capacity factor, k', and the distribution coefficient, P, *cf.*, ref. 15

$$\log k' = \log P + \log \varphi \tag{1}$$

where $\varphi(x) = V_s/V_m$, V_s and V_m are the volumes of the stationary and mobile phases (the latter of composition x, as fraction, v/v of organic component) and k' is a function of the dead volume of the column, V_0, and of the elution volume, V_R:

$$k' = (V_R - V_0)/V_0 \tag{2}$$

According to the general equation of Collander[16] between the distribution coefficients, P_a and P_b, of a single species in two different aqueous organic systems, a and b

$$\log P_a = m \log P_b + \tilde{n} \tag{3}$$

where m and $\tilde{n}$ are two characteristic parameters of the working systems. Since P_a and P_b are referred to each organic solvent–water system, the Collander equation involves the use of pure water as the mobile eluting phase in any reversed-phase high-performance liquid chromatography (RP–HPLC) experiment designed to establish correlations between the distribution in a column and the distribution in an aqueous organic system. As this is not practical, efforts have been made to palliate the problem by measuring k' under different mobile phase conditions, then extrapolating to a zero concentration of organic solvent[17]. However, the function $\log k' = f(x)$ is complicated[18,19] and even more so close to 100% water[18]; for most authors this kind of linear extrapolation involves considerable error[20]. As a more feasible solution, the measurement of distribution coefficients by correlation with capacity factors corresponding to conventional mobile phases has been proposed.

A result of such methodology has been the interest in the correct measurement of dead volume. Different techniques have been advanced.

(a) Determination by direct weighing[21], which involves an appreciable degree of error derived from the measurement of a small magnitude such as the difference between much larger terms. The dead volume thus determined is generally considered

as an extreme value, $V_{0_{max}}$, since the amount of organic component that can solvate the stationary phase is not taken into account[22].

(b) Injection of organic or inorganic salts under particular elution conditions[23–25]. This also has drawbacks, mainly owing to repulsion effects between charges that alter the elution time, and hence the measurement of V_0.

(c) Injection of isotopic species of some of the species not retained in the elution; this method requires the use of a differential refractive index detector. It is a technique that has been widely reported, *cf.*, refs. 26–28, and has shown that the value of V_0 does not remain constant with different mobile phase compositions[26] and normally shows a minimum in the range 40–60% of methanol or acetonitrile[29].

In 1985, Knox and Kaliszan[30] reported that none of these methods leads to a rigorous definition of the thermodynamic dead volume and proposed as such the total volume of the eluents present in the column packing; they used labelled isotopic samples and monitored the elution with a scintillation counter.

METHODS

Starting from the extrathermodynamic correlation between the distribution coefficient, P, and the simplest structural index, the number of carbon atoms of the terms of an homologous series, n_c[1]

$$-(\Delta G)/RT = \ln P = An_c + B \tag{4}$$

and taking into account eqns. 1 and 2, one easily arrives at the expression

$$V_R = V_0(1 + k'_0 e^{\alpha' n_c}) \tag{5}$$

where k'_0 represents the capacity factor of the parent molecule of the series. The third parameter, α', is directly related to the selectivity of the system, α, and, in terms of energy, corresponds to the increase in the variation of the free energy associated with the distribution phenomenon during column elution of the two solutes of the homologous series differentiated by one carbon atom in their chains:

$$\alpha' = \ln \alpha = \ln \frac{k'_n}{k'_{n-1}} = \frac{1}{RT}(\Delta G_{n-1} - \Delta G_n) = \frac{-\Delta(\Delta G)}{RT} \tag{6}$$

After searching for correlations of the kind shown in eqn. 4, our aim was to study the relationships of k'_0 and α with the composition of the eluting phase in order to apply these to the calculation of distribution coefficients, with the consequent advantages not only in speed of operation, but also in safety when working with very dangerous species such as the nitrosamines.

In order to investigate the variation in α' with the composition of the eluting phase, the empirical methodology developed by Jandera *et al.*[27] was employed. According to this, reversed-phase chromatography is mainly controlled by the interactions occurring in the mobile phase, such that the energy change during the transference of 1 mol of solute from this phase to the stationary phase will be given by

$$-\Delta E = E^{P}_{(M-M)} - E^{P}_{(M-X)} \tag{7}$$

where the first term on the right is directly associated with the energy of cohesion between the molecules of the mobile phase, M, and the second term corresponds to the solute (X)–mobile phase interaction.

According to this model, the polar interaction energy between two molecular species can be expressed as a function of a pair of parameters characteristic of each: I_i, the interaction index, which is constant for each solute, and C_i, the interaction coefficient, which depends on the nature of the molecular species:

$$E^{\mathbf{P}}_{(\mathrm{A-B})} = C_{\mathrm{A}} I_{\mathrm{A}} C_{\mathrm{B}} I_{\mathrm{B}} \tag{8}$$

According to this, the free energy associated with the distribution of 1 mol of solute of n carbon atoms can be expressed as

$$\Delta G_n = -(C_{\mathrm{M}}^2 I_{\mathrm{M}}^2 - C_{\mathrm{M}} I_{\mathrm{M}} C_{\mathrm{X}_n} I_{\mathrm{X}_n}) V_{\mathrm{X}_n} \tag{9}$$

where X_n represents a solute of n carbon atoms and V_{X_n} its molar fraction.

The third and last supporting logistic aspect taken from the methodology of Jandera to develop our treatment lies in expressing the interaction index of the mobile phase as a linear function of its composition, x (in molar fraction of organic compound):

$$I_{\mathrm{M}} = (1 - x) I_{\mathrm{wat}} + x I_{\mathrm{org}} \tag{10}$$

On the basis of the ideas put forward, α' can be expressed as:

$$\alpha' = -\frac{\Delta(\Delta G)}{RT} = \frac{C_{\mathrm{M}}^2 I_{\mathrm{M}}^2}{RT}(V_{\mathrm{X}_n} - V_{\mathrm{X}_{n-1}}) - \frac{C_{\mathrm{M}} I_{\mathrm{M}}}{RT}(C_{\mathrm{X}_n} I_{\mathrm{X}_n} V_{\mathrm{X}_n} - C_{\mathrm{X}_{n-1}} I_{\mathrm{X}_{n-1}} V_{\mathrm{X}_{n-1}}) \tag{11}$$

Since the interaction coefficient, C_{X_i}, is constant within an homologous series (the presence of the same functional group), $C_{\mathrm{X}_n} = C_{\mathrm{X}_{n-1}} = C_{\mathrm{X}'}$, and taking into account that both the molar volume and the interaction index are a linear function of the number of carbon atoms, n_c[28]

$$\alpha' = \alpha_0 - \alpha_1 x + \alpha_2 x^2 \tag{12}$$

where

$$\alpha_0 = \frac{C_{\mathrm{M}}}{RT} \cdot I_{\mathrm{wat}}[(C_{\mathrm{M}} I_{\mathrm{wat}} \Delta V_{\mathrm{X}}) - C_{\mathrm{X}}(I_{0\mathrm{X}} \Delta V_{\mathrm{X}} + V_{0\mathrm{X}} \Delta I_{\mathrm{X}})]$$

$$\alpha_1 = \frac{C_{\mathrm{M}}}{RT}(I_{\mathrm{wat}} - I_{\mathrm{org}})[2 C_{\mathrm{M}} \Delta V_{\mathrm{X}} I_{\mathrm{wat}} - C_{\mathrm{X}}(I_{0\mathrm{X}} \Delta V_{\mathrm{X}} + V_{0\mathrm{X}} \Delta I_{\mathrm{X}})]$$

$$\alpha_2 = \frac{C_{\mathrm{M}}^2}{RT}(I_{\mathrm{wat}} - I_{\mathrm{org}})^2 \Delta V_{\mathrm{X}} \tag{13}$$

where V_0 and I_0 correspond to the parent molecule.

Applying the same consideration to eqn. 1, one obtains

$$\ln k'_0 = \ln \varphi(x) + \beta_0 - \beta_1 x + \beta_2 x^2 \tag{14}$$

where:

$$\beta_0 = \frac{C_M}{RT} \cdot V_{0X}(C_M I_{wat}^2 - C_X I_{0X} I_{wat})$$

$$\beta_1 = \frac{C_M}{RT} \cdot V_{0X}(I_{wat} - I_{org})(2C_M I_{wat} - C_X I_{0X})$$

$$\beta_2 = \frac{C_M^2}{RT} \cdot V_{0X}(I_{wat} - I_{org})^2 \tag{15}$$

From expressions 12 and 14 one can write:

$$\ln k' = \ln \varphi(x) + (\beta_0 - \beta_1 x + \beta_2 x^2) + (\alpha_0 - \alpha_1 x + \alpha_2 x^2) n_c \tag{16}$$

Eqn. 16 now allows us to express the capacity factor as a function of n_c and of the mobile phase composition.

EXPERIMENTAL

A Model 500A HPLC chromatograph (Konik Instruments, Barcelona, Spain) was used with a Rheodyne 20-μl injector, a guard column Phase Sep Spherisorb S5CN (5 cm × 4.6 mm; 5 μm) just before a column of Phase Sep. Spherisorb S5CN (10 cm × 4.6 mm; 5 μm) (a type of column frequently employed in the chemistry of N-nitroso compounds[31,32]) and a double beam VIS–UV 757 detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.). The detector signal was programmed with an SP 4290 integrator (Spectra Physics, San José, CA, U.S.A.).

The mobile phase was prepared by mixing water (doubly distilled in glass with addition of potassium permanganate) with the organic solvents at the required volume ratio. Methanol, acetonitrile and isooctane were "HPLC solvents" from Farmitalia, Carlo Erba (Milan, Italy).

The homologous series of N-nitrosamines were all from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Experiments for determining the distribution coefficients of N-nitrosamines were carried out by analyzing the composition of the aqueous phase by an HPLC technique, working at 30°C; the P values, expressed as quotients of molar fractions, were the means of six determinations for each N-nitrosamine.

Retention volumes, V_R, were calculated as the arithmetic means from three injections.

DATA TREATMENT

The V_0, k'_0 and α' values were obtained as fitting parameters in the optimization

TABLE I

RETENTION VOLUMES (TIME, s) OF N-NITROSAMINES IN METHANOL–WATER AND ACETONITRILE–WATER MOBILE PHASES

Guard column: Phase Sep Spherisorb S5CN (5 μm, 5 cm). Column: Phase Sep Spherisorb S5CN (5 μm, 10 cm). Solvent flow-rate: 1 ml min^{-1}.

Nitrosamine	*% (v/v) Methanol*							*% (v/v) Acetonitrile*							
	30	*35*	*40*	*45*	*50*	*55*	*60*	*20*	*25*	*30*	*35*	*40*	*45*	*50*	*55*
NDMA	3.59	3.48	3.41	3.36	3.31	3.25	3.19	3.42	3.37	3.31	3.27	3.21	3.19	3.13	3.09
NMEA	4.07	3.83	3.67	3.57	3.46	3.35	3.27	3.75	3.65	3.54	3.46	3.37	3.32	3.23	3.15
NDEA	4.85	4.35	4.04	3.85	3.67	3.48	3.35	4.27	4.07	3.87	3.73	3.58	3.49	3.36	3.24
NMPA	4.81	4.34	4.05	3.84	3.66	3.49	3.35	4.26	4.05	3.87	3.73	3.58	3.49	3.35	3.23
NDPA	8.27	6.35	5.36	4.73	4.23	3.85	3.56	6.32	5.53	4.92	4.53	4.15	3.93	3.65	3.43
NMBA	6.24	5.24	4.63	4.26	3.93	3.65	3.45	5.14	4.71	4.35	4.11	3.85	3.69	3.49	3.32
NEBA	8.46	6.51	5.42	4.79	4.29	3.85	3.56	6.44	5.62	4.96	4.57	4.16	3.93	3.65	3.42
NPBA	12.33	8.60	6.65	5.56	4.73	4.11	3.71	8.61	7.00	5.85	5.17	4.55	4.24	3.85	3.54
NDBA	19.71	12.27	8.67	6.76	5.45	4.47	3.89	12.76	9.44	7.23	6.06	5.09	4.59	4.05	3.67

of eqn. 5 using the Gauss–Newton algorithm with statistical weights, ω_i, accumulated over the dependent variable. Taking into account that the main source of error in the retention time is not so much in the flow-rate but rather in the phenomenon of retention itself in the stationary phase, and that less strongly retained species should not be weighted as much as those which are strongly retained, we employed weighting factors inversely related to the difference in retention time in the stationary phase ($V_R - V_0$), *i.e.*, the difference between the retention time and the apparent dead volume. In these terms the function to be minimized is:

$$S = \sum_i [V_{R_i} - V_0(1 + k'_0 e^{\alpha' n_{c_i}})]^2 \omega_i \tag{17}$$

Expressions similar to this have been used by different authors[25,29].

RESULTS AND DISCUSSION

The methodology described above was applied to a series of nine nitrosamines: N-nitrosodimethylamine (NDMA), N-nitrosomethylethylamine (NMEA), N-nitrosodiethylamine (NDEA), N-nitrosomethyl-*n*-propylamine (NMPA), N-nitrosodi-*n*-propylamine (NDPA), N-nitrosomethyl-*n*-butylamine (NMBA), N-nitrosoethyl-*n*-butylamine (NEBA), N-nitroso-*n*-butyl-*n*-propylamine (NPBA) and N-nitrosodi-*n*-butylamine (NDBA), after verifying fulfilment of correlation[5] with different organic solvents. Fig. 1 shows this correlation when working with isooctane.

Table I shows the values found for the retention volumes in different mobile phases. On subjecting these results to the above-described fitting (eqns. 5 and 17), the optimum parameters shown in Table II were obtained. Regarding the results, it is interesting that the contribution of the carbon chain to the value of the distribution coefficient is independent of its position (see Fig. 1: two pairs of linear isomers whose coefficients coincide have been included). The data shown in Table I point to a similar kind of behaviour with respect to the elution times of these isomers. Although in the case of NDPA and NEBA (both with $n_c = 6$) the retention of NDPA in weak mobile phases is less than their NEBA, suggestive of a subtle effect of the position of the

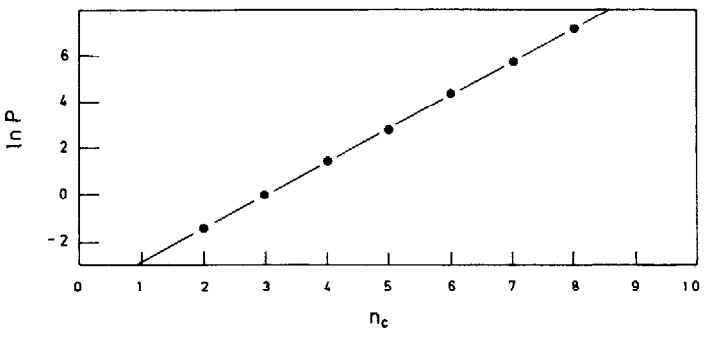

Fig. 1. Correlation between experimental isooctane–water distribution coefficients (ln P) and the number of carbon atoms, n_c, in a series of nitrosamines: 2 (NDMA); 3 (NMEA); 4 (NMPA and NDEA); 5 (NMBA); 6 (NDPA and NEBA); 7 (NPBA); 8 (NDBA).

TABLE II

VALUES OF V_0, $\ln k'_0$ AND α' DETERMINED BY LINEAR REGRESSION (EQNS. 5 AND 17) OF EXPERIMENTAL RETENTION VOLUMES (TIME, s) SHOWN IN TABLE I AGAINST THE NUMBER OF CARBON ATOMS, n_c, BY THE GAUSS–NEWTON ALGORITHM

	% (v/v) Methanol						
	30	*35*	*40*	*45*	*50*	*55*	*60*
V_0	2.97 ± 0.02	2.94 ± 0.02	2.90 ± 0.02	2.89 ± 0.02	2.89 ± 0.02	2.87 ± 0.02	2.85 ± 0.02
$-\ln k'_0$	2.64 ± 0.04	2.60 ± 0.04	2.54 ± 0.04	2.50 ± 0.04	2.51 ± 0.04	2.48 ± 0.05	2.49 ± 0.05
α'	0.543 ± 0.006	0.467 ± 0.005	0.401 ± 0.005	0.347 ± 0.004	0.296 ± 0.004	0.237 ± 0.005	0.184 ± 0.005

carbon atoms on the chain, the fact that the elution times are coincident in strong mobile phases ($\geqslant 55\%$ methanol) points to the notion that such differences might simply be due to the optimum conditions (with higher resolving power of the column) when working with mobile phases with high percentages of water and long elution times. Furthermore, the relative variation of the optimized values of V_0 with the eluent composition is concordant with the fact[33] that the amount of organic component adhering to the stationary phase varies as a function of the nature and percentage of this phase.

The optimized values of $\alpha'(x)$ were fitted by the least squares algorithm to a second degree polynomial expression (eqn. 12); the results are shown in Table III.

A usual practice is the first degree reduction of equations of this kind[28,34,35]. To analyze this kind of protocol, our experimental data were fitted to a linear function (Table III). Application of the F test shows that the second degree is significant with respect to the first degree at the 95% level when working with methanol and at 99% when working with acetonitrile. We believe that these results preclude the possibility of carrying out this simplification that is normally done in lower composition ranges of the mobile phase and at high concentrations of the organic component[28] (see Figs. 2 and 3).

Moreover, the results obtained confirm the expected effect (eqn. 13) of the

TABLE III

VALUES OF α_0, α_1, α_2 DETERMINED BY THE LEAST SQUARES ALGORITHM AT USING THE SECOND DEGREE POLYNOMIAL EXPRESSION (EQN. 12) AND α_0, α_1 DETERMINED BY THE SQUARE ALGORITHM OF THE LINEAR FUNCTION (FIRST DEGREE)

Application of the F test shows that the second degree is significant with respect to the first at the 95% (methanol) and 99% levels (acetonitrile).

Degree		*Methanol*	*Acetonitrile*
1	α_0	0.88 ± 0.01	0.67 ± 0.03
	α_1	$(-1.17 \pm 0.03) \cdot 10^{-2}$	$(-1.04 \pm 0.07) \cdot 10^{-2}$
	Residual	$4.08 \cdot 10^{-4}$	$2.85 \cdot 10^{-3}$
2	α_0	1.02 ± 0.05	0.88 ± 0.02
	α_1	$(-1.8 \pm 0.2) \cdot 10^{-2}$	$(-2.2 \pm 0.1) \cdot 10^{-2}$
	α_2	$(7.4 \pm 2.4) \cdot 10^{-5}$	$(1.6 \pm 0.2) \cdot 10^{-4}$
	Residual	$1.18 \cdot 10^{-4}$	$1.50 \cdot 10^{-4}$

% (v/v) Acetonitrile							
20	*25*	*30*	*35*	*40*	*45*	*50*	*55*
2.92±0.02	2.86±0.02	2.76±0.02	2.68±0.02	2.58±0.03	2.57±0.03	2.48±0.04	2.59±0.03
2.75±0.05	2.55±0.05	2.30±0.05	2.10±0.04	1.87±0.06	1.82±0.07	1.64±0.08	1.92±0.09
0.490±0.006	0.419±0.006	0.346±0.005	0.291±0.005	0.230±0.006	0.197±0.007	0.148±0.006	0.130±0.007

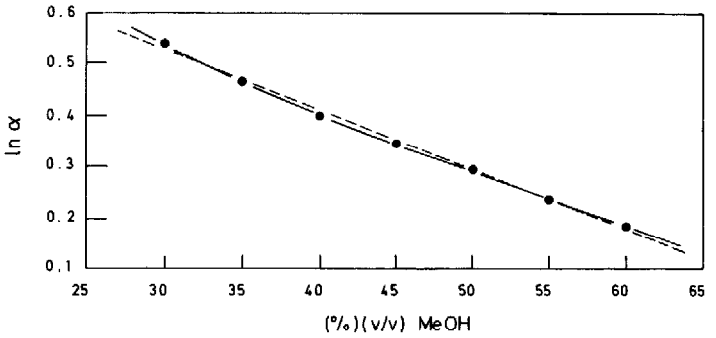

Fig. 2. Plots of the ln α of eqn. 5 against the concentration, x (%, v/v), of methanol in the mobile phase for the homologous series of nitrosamines. Fitting of data to a second degree polynomial expression (———) eqn. 12) and a linear function (---------).

strength of the organic solvent on the second degree term (Table III), since methanol, which is weaker, results in less curvature.

Another interesting aspect is that, according to the interaction index model, the profile of the function relating the values of ln k'_0 with those of the mobile phase composition should be quadratic (like that of ln α) as long as the relationship between the phases is considered constant. In our case it was not possible to observe this kind of

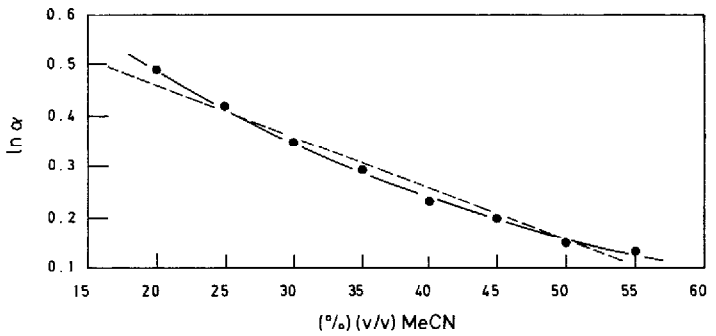

Fig. 3. Plots as in Fig. 2 but for acetonitrile in the mobile phase.

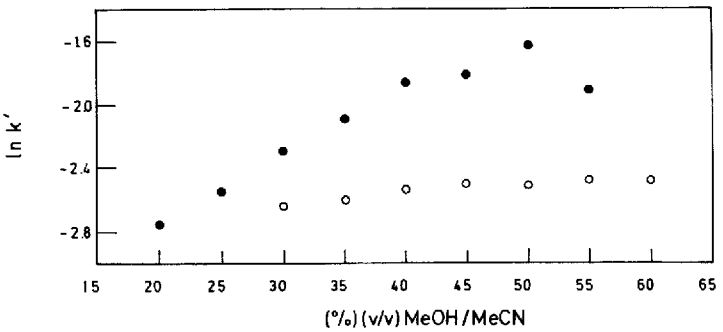

Fig. 4. Logarithmic plots of capacity factors k', of N-nitrosamines against the concentration, x (%, v/v), of methanol–water (○) and acetonitrile–water (●) as the mobile phase on a Phase Sep. Spherisorb S5CN (5 μm) column.

behaviour; by contrast, we observed a variation depending on the sign of the derivative of the dead volume. Thus, as V_R decreases, in φ (and hence in k'_0) should increase, as is shown in Fig. 4. As a result of such findings, we believe that the function φ cannot be considered constant with respect to x (at least under the working conditions used in this study), and therefore there is no point in attempting to fit the data to a polynomial equation, whether of first or second degree.

Using the optimized values of V_0 the k' values were calculated are represented logarithmically against the number of atoms in the carbon chain. Figs. 5 and 6 reveal similar correlations to those obtained with the distribution coefficient, together with another aspect that is worthy of note. This is the appearance of a convergence zone in the sheaf of straight lines. Some authors[28,36] consider this to be characteristic of the homologous series and of each mixture of the mobile phase. However, on recalling the foregoing considerations, the presence of this convergence zone implies that at a certain value, n^*, of n_c, the value of the capacity factor is independent of the mobile

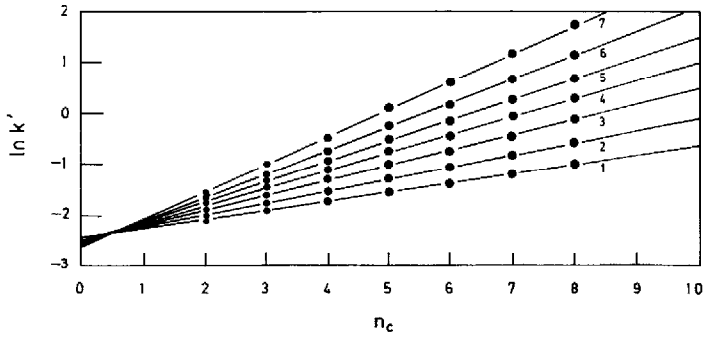

Fig. 5. Logarithmic plots of capacity factors, k', of N-nitrosamines against the numbers of carbon atoms in methanol–water containing 60 (1), 55 (2), 50 (3), 45 (4), 40 (5), 35 (6) and 30% (v/v) (7) of methanol.

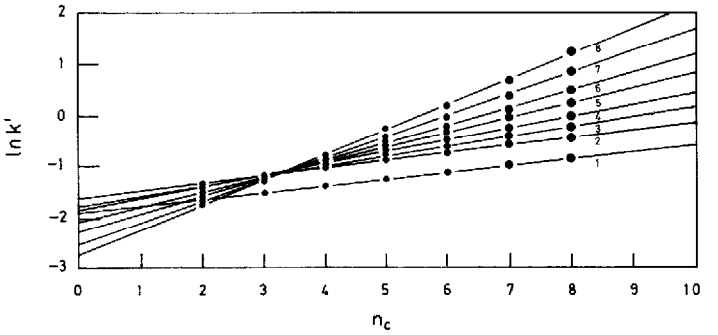

Fig. 6. Logarithmic plots of capacity factors, k', of N-nitrosamines against the numbers of carbon atoms in acetonitrile–water containing 55 (1), 50 (2) 45 (3), 40 (4), 35 (5), 30 (6), 25 (7) and 20% (v/v) (8) of acetonitrile.

phase composition, k^* thus being constant

$$\ln k^* = \ln k'_0(x) + n_c^* \ln \alpha(x) = \text{constant} \tag{18}$$

in other words:

$$\ln k^* = \ln \varphi(x) - \frac{\Delta G_0}{RT} - \frac{\Delta(\Delta G_0)}{RT} \cdot n_c^* = \text{constant} \tag{19}$$

Accordingly, we feel that the appearance of this point (or rather zone) of intersection may simply be the result of a compensation of the variations of the three summands of eqn. 19 and as consequence we cannot attribute to it any well defined physical significance. However, this opinion is not *sensu stricto*, in disagreement with the idea that the intersection zone is characteristic of the homologous series. Nevertheless it is no less true that, while we cannot find the explicit characteristic of the function $\varphi(x)$, we shall not be able to give to the intersection point any other meaning.

CONCLUSIONS

The values of the column dead volume, V_0, for a given column–mobile phase system can be determined from the experimental retention volumes, V_R, of an homologous series of compounds for which there is a free energy correlation between their distribution coefficients and some structural index, such as the number of carbon atoms n_c. The variations occurring in the values of V_0 thus calculated with the mobile phase composition are in excellent agreement with the laboratory results.

Application of the interaction index model to reversed-phase liquid chromatography yields a quadratic equation for the logarithm of the selectivity ($\ln \alpha$), in good concordance with the experimental results. In none of the cases studied (methanol, acetonitrile) is it possible to leave out the quadratic term within the concentration range studied.

The variation in k' with x cannot be dealt with as assumed by the interaction index model, since considerable variations of φ with x are detected. Regarding this point, it should be noted that the possible omission of the quadratic term in the $\ln k'/x$

eqn. 16 is only a question of the use to which it is to be put. Thus, although from a rigorous point of view the problem of having no knowledge of the explicit characteristic of the function $\varphi(x)$ is inevitable, when equations like 16 are used for predictive purposes it is clear that the quadratic term can be neglected.

Finally, and according to our own criteria, the existence of a convergence zone in the linear plots of ln k' against n_c for different compositions should not necessarily be understood as a specific property of the series, but rather as a result of a compensation of the terms governing the dependence of the capacity factor on the eluent composition.

ACKNOWLEDGEMENTS

J.C. thanks the Spanish Comisión Interministerial de Ciencia y Tecnología, CICYT, for supporting the research reported as part of an investigation into Structure/Reactivity Correlations in the Chemistry of N-nitroso compounds (Grant No. PA86/0111). Thanks are also due to the referees for their kind comments and suggestions.

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