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ALGORITHMS TO CORRECT GRADIENT SCAN AND TRANSFER RULE PREDICTIONS OF ISOCRATIC RETENTION IN REVERSED-PHASE LIQUID CHROMATOGRAPHY

DAVE P. HERMAN, HUGO A. H. BILLIET* and LEO DE GALAN

Department of Analytical Chemistry, Delft University of Technology, de Vries van Heystplantsoen 2, 2628 RZ Delft (The Netherlands)

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SUMMARY

An efficient means of predicting solute retention in the three common binary eluents used in reversed-phase liquid chromatography is to pre-analyse the sample using an exploratory methanol–water gradient scan and thereafter calculate iso-elutotropic acetonitrile–water and tetrahydrofuran–water compositions via a simple set of transfer rule equations. However, for some samples the single gradient scan and transfer rule equations fail to yield accurate estimates of k' values in these eluents. In this paper a procedure is described by which these occasionally errant initial predictions can be readily corrected by (1) combining the gradient scan data, T_r' , with the first measured isocratic methanol–water retention data, k' , and (2) combining the results of this extended gradient–isocratic procedure with a new, re-evaluated set of transfer rule equations. A step-by-step procedure is proposed by which these correction algorithms may be invoked in a logical manner to define rapidly the limits of the mobile phase search area in an eluent optimization procedure.

INTRODUCTION

Optimization of eluent composition is often the most time-consuming step in the analysis of complex mixtures by reversed-phase liquid chromatography. The four most commonly used selectivity-adjusting solvents for reversed-phase liquid chromatography are methanol, acetonitrile, tetrahydrofuran (THF) and, of course, water. The major steps involved in determining what combination of these four solvents (and in what relative concentrations) will best separate a specific mixture include defining a suitable range of mobile phase compositions over which to search for the eluent optimum, acquiring retention data adequately spanning the chosen range, analysing these data via one of several different methodologies and, finally, from this analysis, calculating the optimum mobile phase composition. In this paper we shall concentrate on the first step in this sequence. Specifically, we wish to discuss methods for rapidly and accurately predicting solute retention behaviour in binary eluents for the purpose of selecting starting conditions for eluent optimization in reversed-phase liquid chromatography.

An increasingly accepted method for estimating the isocratic retention of any solute molecule in the three common binary eluents methanol–water, acetonitrile–water and THF–water is first to pre-analyse the sample using an exploratory methanol–water gradient scan. From measured retention times under gradient elution conditions, capacity factors (k') in methanol–water eluents under isocratic conditions can be estimated¹. It is then up to the analyst to select that methanol–water composition which yields an adequate degree of retention for all sample components, *i.e.*, where the least and most retained solutes fall within an acceptable range (*e.g.*, $1 \leq k' \leq 10$). A means by which to select this k' range based on the component number and the polarity range of the mixture was described previously². The acetonitrile–water and THF–water concentrations which are predicted to yield chromatograms exhibiting the same range of k' values, that is, which are predicted to be iso-elutropic with the previously selected binary reference methanol–water composition, can be calculated using simple “transfer rule” equations¹. A detailed description of the origin and utility of these gradient scan and transfer rule procedures are presented in the Theory and Results and Discussion sections.

Although the isocratic retention behaviour of many solutes can be accurately predicted from the gradient scan and transfer rule equations, for some solutes large deviations between predicted and experimentally measured k' values have been observed³. The purpose of this study was to determine the source(s) of these discrepancies, if and when they occur, and to develop a means by which these occasionally errant first predictions can be readily corrected in an efficient stepwise manner. Most important, from the analyst’s point of view, the correction procedures were designed such that the number of additional required experiments is a minimum.

THEORY

Determination of isocratic retention from gradient elution scans

Theoretical models have been published that allow the interconversion of isocratic and gradient elution data for solutes subjected to linear gradients^{4–6}. For those solutes exhibiting a linear relationship between $\ln k'$ and eluent volume fraction, φ , the following simple equations relate the gradient net retention time, T_r' (the retention time under gradient conditions minus the column void time), to isocratic and experimental parameters:

$$T_r' = 1/Sb \ln [1 + Sbt_0k'(\varphi_i)] \quad (1)$$

for $T_r' \leq T_G$, and

$$T_r' = k'(\varphi_f)t_0 + 1/Sb[k'(\varphi_f)/k'(\varphi_i) - 1] + T_G \quad (2)$$

for $T_r' > T_G$. In these equations, and in all equations to follow, S is the positive slope of the $\ln k'$ vs. φ plot, φ_i and φ_f designate the initial and final volume fractions of binary eluent during the gradient scan, T_G is the time of the gradient program, b is the rate of change of eluent composition with time $[(\varphi_f - \varphi_i)/T_G]$ and t_0 is the column void time. In most instances, all solutes will elute before the end of the gradient programme such that only eqn. 1 need be considered. Hence, if we experimentally determine the slope

and intercept of a solute's $\ln k'$ vs. φ plot, we can directly calculate via eqn. 1 or 2 the solute's retention time when subjected to a $\varphi_i \rightarrow \varphi_f$ linear gradient in T_G min.

The reverse process, in which one measures experimentally the gradient retention time and desires to predict isocratic retention from this single measurement, requires that we have some pre-knowledge of the relationship between the slope and the intercept of the solute's $\ln k'$ vs. φ plot. In the absence of this information we are confronted with the problem of trying to solve a single equation with two unknowns, namely, S and $k'(\varphi_i)$. Fortunately, in the methanol-water eluent system most solutes exhibit an approximately linear relationship between the slopes, S_m , and intercepts, $\ln k'_0$, of their $\ln k'$ vs. φ_m plots^{7,8}. From a study of the slope-intercept relationships for a large number of solutes representing many different functional groups, the following equation was derived⁸:

$$S_m = 2.86 + 0.77 \ln k'_0 \quad (3)$$

By substituting eqn. 3 into eqns. 1 and 2, we can derive a new set of equations where isocratic retention data *in the methanol-water eluent system* can be unambiguously predicted from gradient elution data:

$$T_r' = 1/S_m b \ln (1 + S_m b t_0 \exp\{(1 - 0.77\varphi_i)S_m - 2.86\}/0.77\}) \quad (4)$$

and

$$T_r' = t_0 \exp\{(1 - 0.77\varphi_f)S_m - 2.86\}/0.77 + T_G + 1/S_m b [\exp(S_m\varphi_i - S_m\varphi_f) - 1] \quad (5)$$

These equations are the basis of the method proposed by Schoenmakers *et al.*¹ for predicting isocratic retention from a *single* standard gradient scan. It should be recognized, however, that several implicit simplifying assumptions have been made in the derivation of the T_r' - S_m relationships expressed by eqns. 4 and 5. Several non-ideal processes (instrumental and chromatographic) which are unique to gradient elution chromatography can violate these assumptions and thereby lead to errors in calculated S_m values from gradient elution data. Quarry *et al.*^{9,10} described the many non-ideal processes that may occur within the column or originate from the gradient equipment and, in some instances, they derived expressions to correct gradient elution data for these non-ideal effects. In this paper we shall concern ourselves only with those effects which have the potential to degrade the ability of eqns. 4 and 5 to predict isocratic retention behaviour from a preliminary gradient elution experiment. The major non-ideal processes which may occur are (in increasing order of importance):

- (1) solvent demixing caused by preferential adsorption of methanol by the non-polar stationary phase;
- (2) gradient delay as a result of the finite extra-column volume between the pump heads and the column inlet, which in turn causes pre-elution of solutes before the gradient has reached the top of the column;
- (3) gradient profile distortion due to dispersion in gradient mixers, pulse dampers, connecting tubing, etc., which can cause the gradient programme to become non-linear;

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most solutes will be eluted only during that part of the gradient which varies linearly with time. Thus, under these experimental conditions, the gradient linearity assumption implicit in eqns. 4 and 5 is obeyed.

The major source of variations in eluent flow-rate (and hence likewise t_0) during the course of a gradient scan is due to the non-ideal mixing behaviour of methanol–water mixtures. This is not the case for low-pressure gradient devices where the mobile phase is first mixed and then pumped. The volume loss incurred on mixing the two neat solvents varies with the mixing ratio, φ_m . The volume of a methanol–water mixture relative to the sum of the volumes of the two neat solvents (V_m/V^{neat}) is related to φ_m according to the empirical equation

$$V_m/V^{\text{neat}} = 0.130\varphi_m^3 - 0.059\varphi_m^2 - 0.071\varphi_m + 1 \quad (6)$$

From this equation we see that V_m/V^{neat} initially decreases with increasing φ_m , reaches a minimum of 0.964 at $\varphi_m = 0.6$ and thereafter increases until it reaches a value of 1 again at $\varphi_m = 1.0$. Hence, the mobile phase linear flow velocity can vary up to 3.6% during the course of a 0–100% methanol–water gradient experiment. Such relatively small deviations in linear flow velocity (and therefore also t_0) can in some instances cause large errors in estimated S_m values from preliminary gradient elution data. This is readily understood when one realizes that all of the gradient elution equations above ($T_r' = f\{S\}$) were derived assuming that t_0 remains constant. This effect therefore needs to be quantitatively accounted for if the derived gradient equations are to remain analytically valid. We correct the observed net retention times of each solute for the effects of varying flow-rate by substituting for t_0 in the gradient equations the following average void times:

$$\bar{t}_0 = L/\bar{U} \quad (7)$$

where L is column length and \bar{U} is the displacement weighted integrated average linear flow-rate of each solute, given by the expression

$$\bar{U} = \frac{\int_0^L u(z)/[1 + k'(z)]dz}{\int_0^L 1/[1 + k'(z)]dz} \quad (8)$$

where z is the relative displacement of the solute down the column and $k'(z)$ and $u(z)$ represent the capacity factor and the instantaneous linear flow-rate, respectively, at the moment the solute reaches position z in the column. In eqn. 8 $u(z)$ can be calculated as

$$u(z) = \frac{L}{t_0^{\text{neat}}}(C_1\varphi_p^3 + C_2\varphi_p^2 + C_3\varphi_p + C_4) \quad (9)$$

where C_1 – C_4 are the regression coefficients given in eqn. 6, t_0^{neat} is the column void time at the flow-rates delivered by the pump(s) when pumping either neat methanol or neat water as eluent and φ_p is the volume fraction of eluent at the pump head. φ_p written as a function of z is given by

$$\varphi_p = \varphi_i + b \left\{ 1/S_m b \ln \left[1 + S_m b \left(\frac{z}{u_0^{\text{neat}}} \right) k'(\varphi_i) \right] + \frac{z}{u_0^{\text{neat}}} \right\} + b\tau_{p \rightarrow c} \quad (10)$$

where u_0^{neat} is simply L/t_0^{neat} and $\tau_{p \rightarrow c}$ is the pump-to-column delay time ($\tau_{p \rightarrow c} = V_{p \rightarrow c}/F$). Finally, we express k' as a function of z using the following equation:

$$k'(z) = \exp \left\{ \ln(k'_0) - S_m \varphi_i - \ln \left[1 + S_m b \left(\frac{z}{u_0^{\text{neat}}} \right) k'(\varphi_i) \right] \right\} \quad (11)$$

After substituting eqns. 8–11 into eqn. 7, we can calculate \bar{t}_0 by numerical integration. The variation in net retention time due to non-ideal mixing of methanol–water mixtures, $\Delta T_{r_{\text{nim}}}$, is thus equal to the difference in calculated T_r' values from eqn. 4 or 5, whichever is appropriate, using $t_0 = \bar{t}_0$ and $t_0 = t_0^{\text{neat}}$. $\Delta T_{r_{\text{nim}}}$ is a solute-dependent correction factor which is a measure of the extent to which non-ideal mixing causes eqns. 4 and 5 to underestimate T_r' when S_m is known, or overestimate S_m when T_r' is known. Having discussed the four instrumental sources for non-ideality, we now turn our attention to the two solute-dependent factors that may have a much greater influence.

Implicit in the mathematics of eqns. 4 and 5 is the assumption that isocratic $\ln k'$ values vary linearly with φ_m . Over a limited range of methanol–water compositions (specifically, those where $1 \leq k' \leq 10$), the $\ln k' - \varphi_m$ function of most solutes is indeed linear. However, it is generally recognized that most solutes do exhibit some degree of $\ln k' - \varphi_m$ curvature when considering the full range of eluent compositions under which they will probably elute during the course of a standard gradient scan. Under such conditions it may become necessary to express the functional dependence of $\ln k'$ on φ_m as a second-order polynomial of the form

$$\ln k' = a\varphi_m^2 + b\varphi_m + c \quad (12)$$

In order to evaluate the extent to which $\ln k' - \varphi_m$ non-linearity affects the ability of eqns. 4 and 5 to predict T_r' when S_m is known (or predict S_m when T_r' is known), we have performed many computational gradient scans wherein the isocratic retention behaviour of 32 solutes was modelled according to eqn. 12. The retention data used for this purpose can be found in ref. 11. T_r' values were calculated by numerical integration for each of the 32 solutes under the assumed experimental conditions of $\varphi_i = 0.1$, $\varphi_f = 1.0$, $t_0 = 2.08$ min and $T_G = 30$ min. The same computational procedure was again performed after having fitted the experimental isocratic retention data between capacity factors of 1 and 10 to a first-order log-linear polynomial rather than eqn. 12 as was done above. The difference in corresponding calculated T_r' values ($\ln k'$ quadratic in φ_m rather than linear) were statistically evaluated and the average deviation in T_r' due to $\ln k'$ curvature, $\Delta T_{r_{\text{curr}}}$, was found to be 0.16 min ($\sigma = 0.076$). Thus, one

concludes that the average curvature of the $\ln k' - \varphi_m$ function of an "average" solute causes the retention times of gradient scan analysed solutes to be greater by 0.16 min than would be predicted from our gradient equations which assume log-linearity. As a first-order approximation, experimental gradient retention times can be corrected for non-log-linearity by simply subtracting 0.16 min from all experimentally determined T_r' values. By applying this "average" correction factor, all the gradient elution $T_r' - S_m$ relationships listed above become statistically more valid. Furthermore, constraints on the isocratic k' values (between 1 and 10) and the newly defined "standard" gradient (see Results and discussion section) have a minimizing influence on the correction.

By far the largest source of error responsible for discrepancies between actual and calculated values of S_m via eqns. 4 and 5 is the assumption that the linear relationship between the slopes and intercepts of all $\ln k' - \varphi_m$ plots is rigorously described by eqn. 3 for *all* solutes. Inspection of the original 32 solute data set from which eqn. 3 was derived (see Fig. 3 in ref. 8) reveals that slopes and intercepts are not perfectly correlated, but rather are slightly scattered about the line of eqn. 3 with a correlation coefficient of 0.98. As a result, the uncertainty (standard deviation) in the intercept of the $S_m - \ln k'_0$ plot, σ_p , is fairly large ($\sigma_p = \sigma_{S_m} = 0.34$). As will be shown later, for other solutes not among the original 32, the difference between the S_m values calculated via eqn. 3 and true actual values can be much larger ($\Delta S_m \gg 2\sigma_p$). In order to correct eqns. 4 and 5 for those solutes not following the slope- $\ln k'_0$ relationship of eqn. 3, we must replace the approximation of eqn. 3 with an exact relationship. By performing one methanol-water isocratic experiment and measuring k' for each of the mixture's j solutes, $k'_{\text{meas},j}$, at volume fractions φ_{meas} , exact relationships between S_m and $\ln k'_0$ become known when these data are combined with information from the initial gradient run. They are

$$S_j = \ln k'_{0j}/\varphi_{\text{meas}} - \ln k'_{\text{meas},j}/\varphi_{\text{meas}} \quad (13)$$

Substituting eqn. 13 (in lieu of eqn. 3) into eqns. 1 and 2 leads to the following equalities which accurately express the $T_r' - S_m$ relationship for *any* solute:

$$T_{r,j}' = 1/S_j b \ln [1 + S_j b t_0 \exp (\ln k'_{\text{meas},j} + S_j \varphi_{\text{meas}} - S_j \varphi_i)] \quad (14)$$

$$T_{r,j}' = t_0 \exp (\ln k'_{\text{meas},j} + S_j \varphi_{\text{meas}} - S_j \varphi_i) + 1/S_j b \exp (S_j \varphi_i - S_j \varphi_i - 1) + T_G \quad (15)$$

Eqns. 14 and 15 thus provide a means for accurately predicting isocratic S_m values (from which $k' - \varphi_m$ functions are readily calculated) for any solute by combining the retention results of a linear gradient scan with those of a single isocratic experiment. Proof will be given in Table II.

Determination of iso-elutropic binary eluents

After having selected the methanol-water elutropic strength, φ_m^* , which produces a chromatogram in which all solutes of the mixture fall within a suitable range of k' values, there remains the problem of predicting retention in the two remaining binary eluents, acetonitrile-water and THF-water. Calculation of equivalent elutropic strength (relative to φ_m^*) for these two binary modifiers can be

performed using the "solvent polarity scale" first described by Snyder¹². All binary eluents with equivalent solvent polarity are to a first approximation assumed to be iso-elutropic. The expression given by Snyder for calculating the solvent polarity index of a binary solvent mixture is

$$P'_{\text{mix}} = \varphi_A P'_A + \varphi_B P'_B \quad (16)$$

where φ_A and φ_B are the volume fractions of solvents A and B and P'_A and P'_B are the polarity index values of the pure solvents. P' values reported for neat water, methanol, acetonitrile and THF are 9.0, 6.6, 6.2 and 4.2, respectively.

An alternative approach for obtaining the compositions of iso-elutropic binaries is that taken by Schoenmakers *et al.*¹. Based on the detailed isocratic retention behaviour of a set of 32 solutes in all three binary eluents, transfer rule equations relating iso-elutropic acetonitrile–water and THF–water volume fractions, φ_{ACN} and φ_{THF} , to specified binary reference methanol–water volume fractions, φ_m^* , were derived. They are as follows:

$$\varphi_{\text{ACN}} = 0.32\varphi_m^{*2} + 0.57\varphi_m^* \quad (17)$$

$$\varphi_{\text{THF}} = 0.66\varphi_m^* \quad (18)$$

Eqns. 17 and 18 represent an *average* of the eluent transfer behaviours of each of the solutes of the data set considered. The scatter about these average predicted values is fairly large such that large deviations between predicted and actual iso-elutropic volume fractions are observed in practice. Therefore, these equations provide only a "first best guess" prediction of equivalent elutropic strength among the three binary eluents. A re-evaluation of these transfer rule equations and a new algorithm for correcting these first predicted iso-elutropic φ_{ACN} and φ_{THF} volume fractions if in error is described below.

EXPERIMENTAL

All gradient elution and isocratic experiments in Tables I and II were performed on equipment consisting of a Model 1090 liquid chromatograph, a 3392A integrator and a 100 × 2.1 mm I.D. Hypersil ODS (5 μm) analytical column, all from Hewlett-Packard (Waldbronn, F.R.G.).

The isocratic elution data in Table IV were collected on equipment from Waters Assoc. (Milford, MA, U.S.A.) and consisted of an M6000 pump, a Model 440 UV detector and a 100 × 8 mm I.D. Nova-Pak C₁₈ Radial-Pak (5 μm) analytical column.

HPLC-grade methanol, acetonitrile and tetrahydrofuran were obtained from Rathburn (Walkerburn, U.K.). Distilled, deionized, organic-free water was prepared in-house using a Milli-Q water-purification system (Millipore, Molsheim, France).

RESULTS AND DISCUSSION

Rapid evaluation of isocratic retention behaviour using the combined gradient–isocratic method

A convenient way for estimating isocratic methanol–water retention behaviour is to run a “standard” gradient as proposed by Schoenmakers *et al.*¹ (*i.e.*, a 0 → 100% methanol linear gradient, $T_G = 15$ min, $t_0 = 125$ s). From the experimentally determined net retention time of each solute, an estimate of the slope (eqn. 4 or 5) and intercept (eqn. 3) of each solute’s $\ln k' - \varphi_m$ function is calculable; however, as described above, serious errors can be introduced.

Of the six non-ideal processes listed above, the first three may be effectively eliminated/minimized by simple adaptation of “standard” experimental conditions. We now recommend that the gradient time should be extended from $T_G = 15$ to 30 min. Solvent demixing and gradient distortion can be minimized in this way.

Errors in predicted isocratic retention behaviour (S_m and $\ln k_0$) can be further minimized by increasing the initial effective eluent composition of the gradient programme from $\varphi_i = 0$ to 0.1. In this way, the often very non-linear $\ln k' - \varphi_m$ behaviour of many solutes at $\varphi_m < 0.1$ can be avoided.

We now recommend the following instrument settings as the new standard conditions for the preliminary gradient scan: 10 → 100% methanol linear gradient, $T_G = 30$ min, $t_0 = 2$ min; the sample injection is delayed until that moment when the volume fraction methanol–water at the column inlet equals 0.1. This injection delay time can be determined using an UV-active tracer.

In the Theory section we described how to correct gradient elution data for the non-ideal effects of varying flow-rate (eqns. 7–11), $\ln k' - \varphi_m$ non-linearity (subtraction of 0.16 min from all experimental T_r' values) and non-perfectly correlated $\ln k' - \varphi_m$ slopes and intercepts (eqns. 13–15). Hence from the preceding discussions one should reasonably conclude that isocratic retention behaviour can be accurately and unambiguously determined by performing at most two chromatographic experiments (one gradient scan followed by one isocratic run) after having applied all of the correction procedures described above. Unfortunately, some ambiguities remain such that the analyst may be required in some rare instances to perform a second isocratic experiment.

The reason for the potential failure of the combined gradient–isocratic method to predict isocratic retention behaviour becomes readily apparent if we plot T_r' as a function of S_m according to eqns. 14 and 15 for solutes exhibiting a wide range of hydrophobicities. For example, in Fig. 1 the relationship between T_r' and S_m is shown for a set of seven hypothetical solutes having isocratic $k'_{\text{meas},j}$ values of 1, 2, 3, ..., 15 at $\varphi_{\text{meas}} = 0.5$ and analysed utilizing an effective 10 → 100% methanol linear gradient, $T_G = 30$ min, $t_0 = 2.08$ min.

These plots show that for solutes with intermediate k' values (3–7) the measured T_r' values can be nearly independent of S_m (between $S_m = 7$ and 20) and, in some instances, can even lead to two analytical solutions for S_m . Hence for some solutes a double value problem exists for which we are unable to determine which one of the two calculated roots correctly describes the slope of the $\ln k' - \varphi_m$ function. Fortunately, this will not often be the case when using the combined gradient–isocratic procedure for the purpose of selecting starting conditions for eluent optimization.

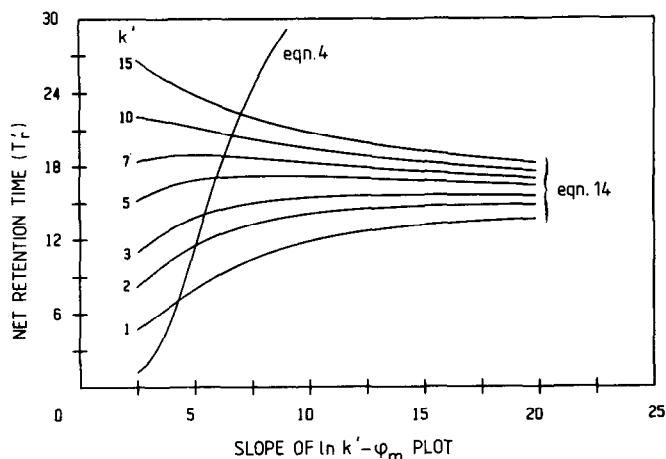


Fig. 1. Plots of gradient scan net retention time as a function of $\ln k' - \varphi_m$ slope according to eqns. 4 and 14 for seven hypothetical solutes exhibiting isocratic k' values of 1, 2, 3, ..., 15 at $\varphi_m = 0.5$. Assumed conditions: 0.1 \rightarrow 1.0 linear methanol-water gradient in 30 min, $t_0 = 2.08$ min.

Also plotted in Fig. 1 is T'_r as a function of S_m according to eqns. 4 and 5 for those ideal solutes obeying the $S_m - \ln k'_0$ linear relationship in eqn. 3. Unlike the case considered above where the $S_m - \ln k'_0$ relationship is determined experimentally by performing a single isocratic experiment and S_m is calculated via eqn. 14 or 15, the eqn. 4 or 5 solution for S_m will be unique and independent of solute hydrophobicity at any measured T'_r value. Although eqns. 4 and 5 will always lead to unique solutions for S_m , the error in these calculated values can be unacceptably large owing to the inadequacy of the eqn. 3 approximation. An efficient and therefore recommended approach for accurately predicting the isocratic retention behaviour of the least and most retained solutes in a mixture is the following:

(1) perform the standard *effective* 10 \rightarrow 100% methanol linear gradient scan and apply eqns. 3–5 to obtain a first estimate of isocratic retention behaviour ($\ln k' - \varphi_m$);

(2) from the results of the preliminary gradient scan, select an eluent composition, φ_m , in which the k' values of the least and most retained solutes span a suitable range and perform an isocratic experiment at this volume fraction;

(3) combine the isocratic retention results of step 2 with the gradient scan retention results of step 1 and use eqns. 13–15 to determine accurately the $\ln k' - \varphi_m$ function of these two limiting solutes. From these functions, choose a suitable binary reference methanol-water composition, φ_m^* . If a double value solution for S_m is found, a second isocratic experiment must be run so as to allow S_m to be determined unambiguously.

In order to demonstrate the ability of the combined gradient-isocratic method to predict accurately the isocratic retention behaviour for solutes known to deviate strongly from the idealized $S_m - \ln k'_0$ relationship of eqn. 3, a six-component mixture was subjected to a 9.3 \rightarrow 100% methanol-water linear gradient, $T_G = 30.27$ min, $t_0 = 2.03$ min. The absolute retention times of all solutes were recorded and are given

TABLE I

DATA DERIVED FROM THE STANDARD GRADIENT AND THE FIRST ISOCRATIC BINARY METHANOL-WATER COMPOSITION OF SIX SOLUTES

Gradient conditions: 9.3 to 100% methanol in 30.27 min, $t_0^{\text{neat}} = 2.03$ min, $\tau_{p \rightarrow c} = 0.61$.

No.	Solute	Gradient scan data				Isocratic data:
		T_r	T_r' ($T_r - t_0^{\text{neat}}$)	$T_r' - \Delta T_{r_{\text{curv}}}$	$T_r' - \Delta T_{r_{\text{curv}}} - \Delta T_{r_{\text{nim}}}$	$k'_{\text{meas}} (\varphi_m = 0.48)$
1	Benzaldehyde	12.54	10.51	10.35	10.16	1.37
2	Benzyl alcohol	15.19	13.16	13.00	12.79	2.22
3	<i>m</i> -Dinitrobenzene	16.54	14.51	14.35	14.12	3.06
4	Dimethyl phthalate	17.36	15.33	15.17	14.93	3.12
5	Prednisone	19.03	17.00	16.84	16.60	5.26
6	Hydrocortisone	20.14	18.11	17.95	17.76	7.62

in the first data column of Table I. The last three data columns list the uncorrected net retention times ($T_r - t_0^{\text{neat}}$), net retention times corrected for $\ln k' - \varphi_m$ curvature ($\Delta T_{r_{\text{curv}}}$) and net retention times corrected for the effect of flow-rate variation due to non-ideal mixing of methanol and water ($\Delta T_{r_{\text{nim}}}$), respectively. The calculation of uncorrected net retention times and retention times corrected for $\ln k' - \varphi_m$ curvature are straightforward and simply require subtracting t_0^{neat} and 0.16 min, respectively, from all absolute retention time values. However, calculation of the correction factors $\Delta T_{r_{\text{nim}}}$ is more complex because of the need to know the S_m and $k'(\varphi_i)$ values for each individual solute in advance (see eqns. 10 and 11). As a first estimate of these values, one can use the eqn. 3-5 solution for S_m and $\ln k'_0$ where the initial corrected net retention time used in the calculation is $T_r' - \Delta T_{r_{\text{curv}}}$. The additional correction term $\Delta T_{r_{\text{nim}}}$ can then be calculated using eqns. 7-11 and the procedure repeated until $\Delta T_{r_{\text{nim}}}$ converges to a constant value. In practice, it is found that only one iteration is required. As can be seen from the data in Table I, $\Delta T_{r_{\text{nim}}}$ values are solute dependent, and for the solutes studied vary between 0.19 and 0.24 min. From this single gradient scan, an isocratic eluent of 48% methanol in water was predicted from eqns. 3 and 4 to yield a chromatogram in which the k' values of the least and most retained solutes span a suitable range, specifically $k' = 1-10$. The k' values actually found for this isocratic run are listed in the last data column in Table I. Ultimately, of course, these single isocratic k' values allow us to use the combined gradient-isocratic procedure (eqns. 13-15) to verify or improve our estimates of $S_{m,j}$ and $\ln(k'_0)_j$.

Fig. 2 shows plots of gradient scan net retention times as a function of S_m (eqn. 14) for the six solutes listed in Table I. Also plotted is T_r' as a function of S_m using the approximation relationship of eqn. 4. The dashed lines represent the $T_r' - S_m$ function for solutes for which the $S_m - \ln k'_0$ relationship is given not by eqn. 3 but rather by $S_m = 2.86 + 0.77 \ln k'_0 \pm 2\sigma_p$, where $\sigma_p = 0.34$ is the scatter about the eqn. 3 line observed by Schoenmakers *et al.*⁸ The area between the two dashed lines therefore represents the 95% confidence interval range of potential S_m values for any solute which exhibits a gradient scan net retention time of T_r' . The dotted points in Fig. 2 are the points at which corrected T_r' values ($T_r' - \Delta T_{r_{\text{curv}}} - \Delta T_{r_{\text{nim}}}$) intersect the corresponding $T_r' - S_m$ curve for each of the six solutes. Except for solute 3, we see that

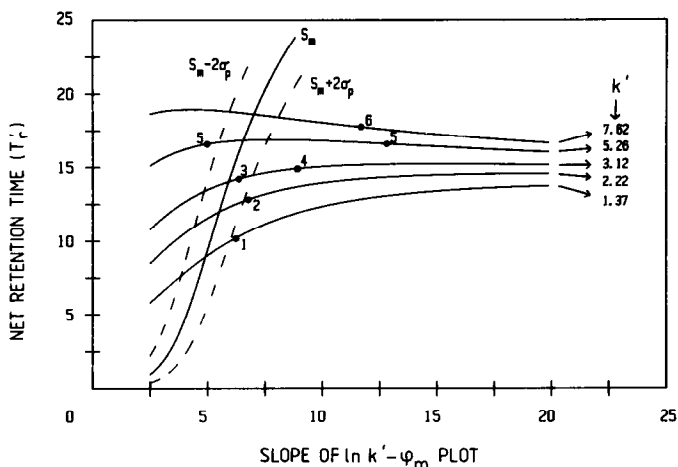


Fig. 2. As Fig. 1, except that T_r' versus S_m plots were generated for the six real solutes listed in Table I whose isocratic k' values at $\phi_m = 0.48$ were measured to be 1.37, 2.22, 3.06, 3.12, 5.26 and 7.62, respectively. Points 1–6 indicate the location where the experimentally determined gradient scan T_r' values (corrected) intersect the T_r' – S_m plot for each solute. For an explanation of the dashed lines at $S_m \pm 2\sigma_p$, see the text. Gradient scan conditions: 0.093 → 1.0 linear methanol–water gradient in 30.27 min, $t_0 = 2.03$ min. Column: 100 × 2.1 mm I.D. Hypersil ODS (5 μm).

all S_m values calculated via eqn. 14 fall outside this 95% confidence interval range. We should warn the reader at this point that the retention behaviour of these particular solutes is not typical, but rather we have deliberately selected these solutes because of their non-ideal behaviour with respect to the correlation between the slopes and intercepts of their $\ln k'$ – ϕ_m plots. These solutes thus serve well to demonstrate the extent to which the combined gradient–isocratic procedure can correct errant first predictions of isocratic retention behaviour obtained via the single gradient scan approach.

Table II gives the S_m values calculated via the original gradient scan procedure

TABLE II

DETERMINATION OF $\ln k'$ – ϕ_m SLOPES FOLLOWING THE SINGLE GRADIENT SCAN AND THE COMBINED GRADIENT SCAN–ISOCRATIC ALGORITHMS

Experimental conditions: 9.3 to 100% methanol in water in 30.27 min, $t_0^{\text{meat}} = 2.03$ min.

Solute	Slope calculated via original gradient scan procedure (WOC)*	Slope calculated via original gradient scan procedure (WC)*	Slope calculated via gradient–isocratic procedure (WC)*	Experimentally determined slope from many isocratic experiments
Benzaldehyde	5.22	5.15	6.14	6.45
Benzyl alcohol	5.74	5.66	6.65	7.03
<i>m</i> -Dinitrobenzene	6.02	5.94	6.26	6.56
Dimethyl phthalate	6.20	6.11	8.79	9.57
Prednisone	6.60	6.50	4.90, 12.77	12.09
Hydrocortisone	6.88	6.78	11.56	12.20

* WC and WOC designate *With* and *WithOut* Correcting gradient scan retention times for the effects of $\ln k'$ curvature ($\Delta T_{r_{\text{curv}}}$) and non-ideal methanol–water mixing ($\Delta T_{r_{\text{nm}}}$).

(eqn. 4) and via the combined gradient–isocratic procedure (eqn. 14). In addition, a separate and independent set of isocratic experiments were performed in order to determine exact S_m values for each of the six solutes studied. These exact values, with which all other calculated S_m values should be compared, are given in the fourth data column in Table II. The first two columns list the S_m values calculated from eqn. 4 using uncorrected net retention times ($T_r - t_0^{\text{net}}$) and using corrected T_r' values ($T_r - \Delta T_{r_{\text{curv}}} - \Delta T_{r_{\text{nim}}}$), respectively. Except for solute 3 (*m*-dinitrobenzene), in both instances large discrepancies between calculated and true values are observed (compare columns 1 and 2 with column 4). On the other hand, S_m values determined via the combined gradient–isocratic procedure with T_r' correction (column 3) are seen to be in much more reasonable agreement with the true values listed in column 4. A single anomaly occurs in the case of solute 5 (prednisone). As can be seen in Fig. 2, for this particular solute two analytical solutions of S_m are found from eqn. 14. In this single instance, a second isocratic experiment would be needed in order to determine which of the two solutions is indeed correct.

The major conclusion to be drawn from these experimental data is that by combining the results of the first isocratic experiment with the retention data of the preliminary gradient scan, greatly improved estimates of isocratic retention behaviour (S_m and $\ln k'_0$) are indeed obtained relative to the single gradient scan procedure first proposed by Schoenmakers *et al.*¹ for those solutes not closely adhering to the slope–intercept relationship of eqn. 3. An accurate determination of these values is the critical first step in selecting the three limiting iso-elutotropic binary eluent compositions, methanol–water, acetonitrile–water and THF–water, which will serve to define the limits of the parameter space searched during the course of either a ternary or quaternary eluent optimization procedure.

Accurate determination of iso-elutotropic compositions by extension/modification of the existing transfer rule equations

For many solutes, the transfer rule eqns. 17 and 18 fail to yield sufficiently accurate predictions of the acetonitrile–water and THF–water compositions having the same overall retention as a specified binary reference methanol–water eluent³. This result is not surprising when one realizes that these transfer rule equations were derived by comparing the retention behaviour of a limited set of 32 *non-structurally related* solutes in each of the three binary eluent systems¹. Specifically, the transfer rule equations were derived by fitting the retention data of each solute in each eluent system to a log-quadratic function of the form of eqn. 12. From these quadratic equations iso-elutotropic eluents were calculated at all binary reference methanol–water compositions for each solute of the data set. A plot of iso-elutropic φ_{THF} versus φ_m^* calculated in this manner is shown in Fig. 3A, where each curve represents one of the 32 solutes. Transfer rule eqn. 18 was obtained by fitting the best straight line with zero intercept through the data. The intercept of these plots must be exactly zero because the origin in the plot represents 100% water in both eluent systems considered.

Of particular concern is the observation that the curves in Fig. 3A are highly scattered such that errors in iso-elutropic THF–water compositions predicted by eqn. 18 will be substantial and are likely to occur often. More important, at least for the discussion to follow, is the observation that virtually none of the solutes exhibit φ_{THF} versus φ_m^* curves tending towards the origin. For this reason, we decided to re-evaluate

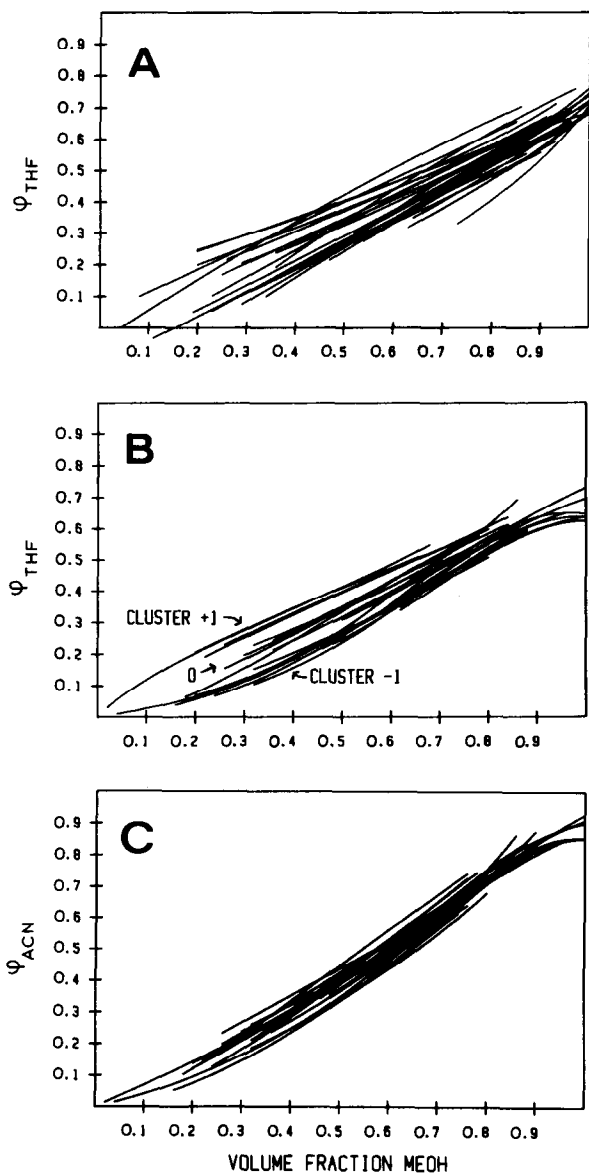


Fig. 3. Plots of iso-elutropic THF-water and acetonitrile-water volume fractions relative to binary reference methanol-water compositions for the 32 solutes listed in ref. 1. Plot A (for THF) was obtained following the procedure in ref. 1 where all isocratic $k'-\phi$ data were fitted to eqn. 12. Plots B (THF) and C (acetonitrile) were derived making use of eqn. 19.

the retention data by forcing the $\ln k'-\phi_{\text{eluent}}$ data for each solute to come to a common intercept at 100% water for each of the three eluent systems *prior* to calculating iso-elutropic compositions rather than *after* calculating these compositions as done previously. For example, Fig. 4 shows $\ln k'-\phi_{\text{eluent}}$ experimental data for dimethyl

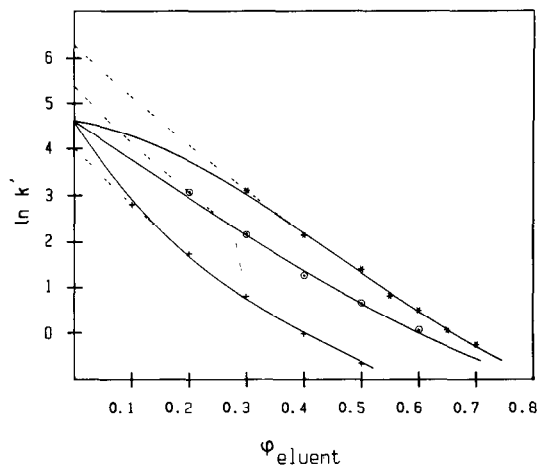


Fig. 4. Dependence of $\ln k'$ for dimethyl phthalate on eluent composition in the three binary eluents methanol-water (*), acetonitrile-water (O) and THF-water (+). The dashed lines were obtained by regression fitting of eqn. 12. The solid curves were obtained by regression fitting of eqn. 19 where the intercept at $\varphi_{\text{eluent}} = 0$ is forced to be in common. Column: 100×8 mm I.D. Nova-Pak C₁₈, Radial-Pak (5 μm).

phthalate in the three binary eluents. Also plotted are quadratic regression equations (eqn. 12) fitted independently to the experimental data of each eluent and third-order log-linear polynomial equations of the form

$$\ln k' = a\varphi_{\text{eluent}}^3 + b\varphi_{\text{eluent}}^2 + c\varphi_{\text{eluent}} + d \quad (19)$$

which have also been fitted to the experimental data but where all three regression equations are forced to come to a common intercept at $\varphi_{\text{eluent}} = 0$; the value of d in eqn. 19 is the same for all three modifiers but different if only quadratic fittings were used. It is clear that quadratic log-linear polynomials are not sufficiently flexible to describe adequately solute retention at eluent compositions near $\varphi_{\text{eluent}} = 0.0$. Therefore, we chose to fit the $\ln k'$ data for each solute of the original data set to eqn. 19 by an iterative multilinear least-squares method and from these equations to calculate iso-elutotropic THF-water and methanol-water volume fractions. Plots of φ_{THF} versus φ_{m}^* evaluated in this slightly different manner are given in Fig. 3B. Although these curves exhibit a great deal of scatter about a central tendency, the data indicate that real solutes tend to cluster into one of three groups for which the intra-group scatter is significantly lower. The clustering has no relation to molecular functional groups. Therefore, rather than attempt to represent all of the data with a single transfer rule equation, we have fitted a simple third-order polynomial with zero intercept through each of the three data clusters in Fig. 3B. The fitting function was of the form

$$\varphi_x = a\varphi_{\text{m}}^{*3} + b\varphi_{\text{m}}^{*2} + c\varphi_{\text{m}}^* \quad (20)$$

where x can represent either THF or ACN. The coefficients a , b and c for each of the three data clusters indicated in Fig. 3B are given in Table III. For a totally unknown

TABLE III

COEFFICIENTS OF THE NEW EMPIRICAL TRANSFER RULE EQUATIONS (SEE EQN. 20) RELATING ISO-ELUOTROPIC ACETONITRILE-WATER AND THF-WATER BINARY ELUENTS TO USER-SPECIFIED BINARY REFERENCE METHANOL-WATER ELUENT COMPOSITIONS

<i>Eluent</i>	<i>Cluster</i>	<i>a</i>	<i>b</i>	<i>c</i>
THF-water	0	-0.420	0.702	0.423
	+1	0.574	-0.906	1.118
	-1	-0.906	1.597	-0.036
Acetonitrile-water	0 σ	-0.490	0.953	0.447
	+4 σ	-0.568	0.799	0.751
	-4 σ	-0.412	1.108	0.143

solute we have no way of predicting in advance into which of the three clusters its φ_{THF} versus φ_m^* function will fall. For this reason we have no choice but to choose the central cluster (cluster 0) as the first best estimate of the iso-elutropic THF-water volume fraction. Thus, we now propose the use of

$$\varphi_{\text{THF}} = -0.420\varphi_m^{*3} + 0.702\varphi_m^{*2} + 0.423\varphi_m^* \quad (21)$$

as the new transfer rule equation in place of eqn. 18.

A similar re-evaluation of the transfer rule equation relating acetonitrile-water to methanol-water was carried out in a similar manner and the iso-elutropic φ_{ACN} versus φ_m^* curves are plotted in Fig. 3C. For this eluent system pair no obvious clustering of solute type is observed. In view of this more ideal transfer behaviour, a single third-order polynomial of the form of eqn. 20 was fitted through all of the data. The coefficients *a*, *b* and *c* are also reported in Table III (refer to the 0 σ values). Therefore, we now propose that eqn. 22 be used in place of eqn. 17 for calculating iso-elutropic acetonitrile-water compositions:

$$\varphi_{\text{ACN}} = -0.490\varphi_m^{*3} + 0.953\varphi_m^{*2} + 0.447\varphi_m^* \quad (22)$$

Also reported in Table III are the coefficients of the regression equations (eqn. 20) fitted through the data points φ_m^* , $\varphi_{\text{ACN}} + 4\sigma$ and φ_m^* , $\varphi_{\text{ACN}} - 4\sigma$, where σ is the standard deviation in φ_{ACN} at each φ_m^* value. In the discussion to follow, the transfer behaviour of solutes between acetonitrile-water and methanol-water will be treated as if real solutes distributed themselves among three data clusters, *i.e.*, between 0 σ , +4 σ and -4 σ . We justify the creation of two "artificial" data clusters at +4 σ and -4 σ based on the following reasoning. First is the observation that a few solutes have recently been found (not part of the original 32 solute data set) which exhibit both positive and negative deviations in φ_{ACN} which fall outside the 99.9 confidence interval range of values represented in Fig. 3C (*i.e.*, at values greater than $\pm 3.7\sigma$). Also, because of the requirement that all φ_{ACN} versus φ_m^* plots must pass through the origin, solutes whose true iso-elutropic φ_{ACN} values differ significantly in the positive (negative) sense from eqn. 22 will have φ_{ACN} versus φ_m^* plots whose instantaneous

slopes, $d\varphi_{\text{ACN}}/d\varphi_m^*$, are substantially larger (smaller) than those calculated from eqn. 22. The importance of the derivative functions $d\varphi_{\text{THF}}/d\varphi_m^*$ and $d\varphi_{\text{ACN}}/d\varphi_m^*$ will become clear shortly.

We now address the problem of how to correct eqns. 21 and 22 if and when they fail to predict correctly, within acceptable error limits, iso-elutotropic THF–water and acetonitrile–water volume fractions relative to a specified binary reference methanol–water composition. Specifically, we wish to know how much to change the eluent composition, φ_x , predicted by eqn. 21 or 22 if a specific experimentally determined capacity factor, k'_x , in eluent φ_x differs significantly from its capacity factor, k_m^* , in the binary reference methanol–water eluent, φ_m^* . To calculate the change in φ_x required to shift k'_x to the desired value k_m^* , we simply multiply the change in methanol–water composition required to shift k'_x to k_m^* [given by $(\ln k'_x - \ln k_m^*)/S_m$] by the derivative function $d\varphi_x/d\varphi_m^*$ evaluated at φ_m^* . Therefore, to a good first approximation, by combining the isocratic retention data of the first acetonitrile–water or THF–water chromatogram with gradient scan determined values of S_m , φ_m^* , and k_m^* in the methanol–water eluent system, a more accurate prediction of iso-elutotropic eluent, φ'_x , can be calculated according to the equation

$$\varphi'_x = \varphi_x + d\varphi_x/d\varphi_m^*(\ln k'_x - \ln k_m^*)/S_m \quad (23)$$

where the derivative $d\varphi_x/d\varphi_m^*$ is first evaluated at φ_m^* using eqn. 21 or 22. This first calculated value of φ'_x is then used to determine the relative placement of the solute under consideration among the three φ_x versus φ_m^* data clusters and, from this, a new weighted average derivative is calculated. For example, assume for THF–water that it is found that our first estimate of φ'_{THF} falls between the φ_{THF} value calculated using the cluster 0 coefficients in Table III and φ_{THF} evaluated using cluster –1 coefficients. In this instance we would calculate the derivatives $d\varphi_x/d\varphi_m^*$ from both cluster 0 and cluster –1 equations and then use an average derivative weighted according to the relative distance of φ'_{THF} between the two clusters to calculate a new φ'_{THF} value from eqn. 23. This new φ'_{THF} is then used to calculate a new weighted average derivative and the iteration procedure continued until φ'_{THF} converges to a constant value. In practice it is found that two iterations are usually sufficient.

In order to test the validity of the eqn. 23 iteration procedure we have collected isocratic retention data for five solutes at several binary reference methanol–water compositions, φ_m^* , and at acetonitrile–water and THF–water compositions predicted to be iso-elutotropic with φ_m^* according to eqns. 17, 18 and 21–23. The k' values of the five solutes studied in these supposedly iso-elutropic eluents are given in Table IV.

Of the five solutes listed, the two steroids prednisone and hydrocortisone are of particular interest because they both exhibit very non-ideal eluent transfer behaviour. For example, from Table IV it is seen that k' for hydrocortisone in 40% methanol was measured to be 9.7. The volume fractions acetonitrile–water predicted to be iso-elutotropic with $\varphi_m^* = 0.4$ according to eqn. 17 and 22 are 0.279 and 0.300, respectively. The respective k' values at these two eluent compositions were measured and found to be 2.7 and 1.9. Clearly, estimates of iso-elutotropic acetonitrile–water composition from eqns. 17 and 22 are grossly in error for this particular solute such that the capacity factors obtained in these solvents differ from the desired target value of 9.7 by a factor of nearly four.

TABLE IV
CAPACITY FACTORS MEASURED IN BINARY ELUENTS PREDICTED VIA THE INDICATED EQUATIONS TO BE ISO-ELUOTROPIC
The values in parentheses refer to the φ_{ACN} or φ_{THF} volume fractions at which the k' values were measured.

Solute	Binary reference		Acetonitrile-water			THF-water		
	φ_m^*	k' at φ_m^*	k' (eqn. 17)	k' (eqn. 22)	k' (eqn. 23)	k' (eqn. 18)	k' (eqn. 21)	k' (eqn. 23)
Benzaldehyde	0.15	6.5	5.5 (.093)	5.9 (.087)	6.6 (.077)	3.2 (.099)	3.7 (.078)	5.1 (.036)
	0.20	4.7	3.8 (.127)	4.0 (.124)	4.8 (.106)	2.5 (.132)	2.9 (.109)	4.0 (.068)
	0.30	2.5	2.0 (.200)	1.8 (.207)	2.6 (.166)	1.6 (.198)	1.8 (.179)	2.3 (.142)
Benzyl alcohol	0.20	9.0	9.5 (.127)	9.7 (.124)	9.0 (.133)	5.1 (.132)	5.9 (.109)	7.4 (.074)
	0.30	4.5	5.2 (.200)	4.9 (.207)	4.5 (.218)	3.3 (.198)	3.8 (.179)	4.3 (.160)
	0.40	2.3	2.9 (.279)	2.6 (.300)	2.4 (.313)	2.2 (.264)	2.3 (.255)	2.3 (.254)
<i>m</i> -Dinitrobenzene	0.20	10.6	18.9 (.127)	19.4 (.124)	8.7 (.223)	19.3 (.132)	22.6 (.109)	10.8 (.211)
	0.40	3.1	5.6 (.279)	4.8 (.300)	2.7 (.379)	7.1 (.264)	7.6 (.255)	3.3 (.353)
	0.50	1.7	2.9 (.365)	2.3 (.401)	1.5 (.454)	4.0 (.330)	3.9 (.335)	1.7 (.423)
Prednisone	0.40	9.9	2.5 (.279)	1.7 (.300)	14.9 (.178)	1.6 (.264)	1.8 (.255)	10.9 (.139)
	0.45	5.4	1.2 (.321)	0.7 (.350)	10.3 (.199)	1.1 (.297)	1.1 (.294)	5.1 (.183)
	0.50	3.0	0.5 (.365)	0.3 (.401)	7.6 (.216)	0.8 (.330)	0.8 (.335)	2.3 (.234)
Hydrocortisone	0.40	9.7	2.7 (.279)	1.9 (.300)	14.6 (.181)	1.8 (.264)	2.0 (.255)	10.3 (.144)
	0.45	5.3	1.3 (.321)	0.8 (.350)	10.2 (.202)	1.3 (.297)	1.3 (.294)	4.9 (.189)
	0.50	3.1	0.6 (.365)	0.3 (.401)	7.8 (.217)	0.9 (.330)	0.9 (.335)	2.4 (.241)

When the single retention datum at $\varphi_{\text{ACN}} = 0.3$ is combined according to eqn. 23 with known S_m , φ_m^* and $k_m'^*$ values to give 0.181 as the new corrected estimate of iso-elutropic acetonitrile, and k' at this eluent composition is measured, we obtain $k' = 14.6$. Although the difference between $k' = 14.6$ and 9.7 is significant, in some applications errors of this magnitude may be tolerable. If a further refinement in iso-elutropic acetonitrile–water composition is desired one may of course use the now known k' values at the first predicted eluent composition, $\varphi_{\text{ACN}} = 0.300$, and at the eqn. 23-corrected eluent composition, $\varphi_{\text{ACN}} = 0.181$, to construct a simple $\ln k' - \varphi_{\text{ACN}}$ plot from which to calculate a better estimate of iso-elutropic φ_{ACN} . However, when considering the transfer behaviour of the five solutes in Table IV as a whole, we observe that the eqn. 23 iteration procedure described above does in most instances predict iso-elutropic acetonitrile–water and THF–water compositions with sufficient accuracy such that additional isocratic experiments in these two eluents need not be performed. From these data we can therefore conclude that the limiting acetonitrile–water and THF–water compositions most suitable for use in a ternary or quaternary eluent optimization procedure can be accurately determined for most mixtures from the results of only one isocratic experiment in each eluent (*i.e.*, from eqn. 23).

GLOSSARY OF TERMS

b	steepness of the gradient $(\varphi_f - \varphi_i)/T_G$
$C_1 - C_4$	regression coefficients in eqn. 9
F	flow through the column
k'	chromatographic capacity factor
k'_0	capacity factor of a solute in 100% water
$k'_{\text{meas},j}$	isocratically measured capacity factors of j solutes at φ_{meas} (a known isocratic methanol–water eluent)
L	column length
$\ln k'$	natural logarithm of the capacity factor
P_{mix}	Snyder's solvent polarity index of a binary mixture
P_A, P_B	Snyder's solvent polarity index for solvents A and B
S	positive slope of the $\ln k' - \varphi$ plot
s	total surface area of sorbent within the column
S_m	positive slope of the $\ln k' - \varphi$ plot for a certain solute in the methanol–water
THF	tetrahydrofuran
T_G	gradient duration time
T_r'	net retention time under gradient conditions
t_0	column void time
t_0^{neat}	column void time at the flow-rates delivered by the pump(s) when pumping either neat methanol or water
\bar{U}	displacement weighted integrated average linear flow-rate
U_0^{neat}	L/t_0^{neat}
V_G	gradient volume; $V_G = T_G F$
V_m	volume of methanol
V_M	effective volume of the gradient mixer
V^{neat}	resulting volume when mixing the two neat solvents methanol and water
$V_{p \rightarrow c}$	volume between the pump head(s) and the column

z	relative displacement of the solute down the column
$\Delta T_{r_{nim}}$	variation in net retention time due to non-ideal mixing of methanol–water mixtures
$\Delta T_{r_{curv}}$	deviation in T_r' due to $\ln k'$ curvature
σ_p	standard deviation of the intercept of the $S_m - \ln k_0$ plot
$\tau_{p \rightarrow c}$	pump to column delay time
φ	volume fraction of organic component in the eluent
φ_f	final volume fraction of organic component
φ_{ACN}	volume fraction of acetonitrile
φ_i	initial volume fraction of organic component
φ_m	volume fraction of methanol
φ_m^*	selected methanol–water isocratic eluent after the gradient run and corrections
φ_{THF}	volume fraction of tetrahydrofuran
φ_p	volume fraction of organic component in the eluent at the pump head
φ_x	volume fraction of tetrahydrofuran or acetonitrile
φ_x^c	corrected volume fraction of tetrahydrofuran or acetonitrile

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