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# USE OF GRADIENT ELUTION FOR RAPID SELECTION OF ISOCRATIC CONDITIONS IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

It is shown that an initial scan of an unknown sample with a gradient elution programme offers a very rapid and easy procedure for deciding whether the sample is suitable for isocratic elution and, if so, for establishing the appropriate binary composition for isocratic separation. The resulting chromatogram is a good first-order approximation for the best possible separation in the given binary mobile phase system. In this way the selection of optimal binary compositions can be greatly accelerated and simplified.

It is also shown that the transfer from one organic modifier to another can readily be made using simple transfer rules. In this way the selectivity can be varied, while the retention remains approximately constant.

## INTRODUCTION

In reversed-phase high-performance liquid chromatography (RP HPLC) the main factor influencing retention and separation is the qualitative and quantitative composition of the mobile phase. For samples compatible with RP HPLC, a proper chromatogram can sometimes be obtained under isocratic conditions and always under gradient elution conditions, where the composition of the mobile phase is varied during the analysis. A proper chromatogram can be defined as a chromatogram in which all of the constituents of the sample are eluted with reasonable retention times.

For routine analysis it is strongly recommended that the application of gradient elution programmes for separations that can also be obtained isocratically be avoided. Unfortunately, optimal isocratic conditions are usually obtained by time-consuming "trial and error" procedures. It should be realized, however, that a single gradient run covers all binary compositions of possible interest to isocratic separation. Consequently, it should be possible to use the results of such a gradient run for the prediction of isocratic retention behaviour. As will be shown in this paper, optimal

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isocratic conditions are then approached more rapidly and conveniently than in current practice.

Thus, after an initial gradient run in a given binary mobile phase system we want to be able to answer the following questions:

(1) Is the sample compatible with RP HPLC?

If the answer is negative, we must switch to another chromatographic system, for which similar schemes can be developed, such as the one presented here for RP HPLC. If the answer is positive, the next question is:

(2) Can a proper chromatogram be obtained under isocratic conditions?

If not, one should try to optimize the gradient programme, using, *e.g.*, the strategy proposed by Snyder and co-workers[1,2]. Alternatively, column-switching techniques may be applied. If the sample is compatible with isocratic elution, the obvious question is:

(3) What is the binary composition that will achieve this goal?

Snyder[3] has suggested a simple way of establishing such a binary composition, based on the local composition of the mobile phase when the solute leaves the column. In this paper we shall try to obtain more accurate results with a graphical procedure based on exact mathematics. If we have arrived at the appropriate binary composition for the original solvents of the gradient run, the actual separation need not be complete for all solutes and a change of modifier might improve the selectivity. Obviously we prefer to avoid another gradient run using a different modifier. Therefore, the fourth and final question to be answered is:

(4) What is the composition of a binary mixture containing another organic modifier, that yields a chromatogram with roughly equal retention times?

The result of a single, initial gradient run is then a binary solvent composition that yields an isocratic chromatogram with optimal selectivity within a reasonable analysis time.

In this paper we describe a convenient procedure for answering the four questions posed above. This procedure is outlined in Fig. 1. After an initial gradient we should decide whether the sample is compatible with the chromatographic system and whether isocratic elution is possible. A negative answer to any of these questions will force us to abandon the present system for separation development. If both questions are answered positively, we enter a loop in which we can try to separate the sample with a series of organic modifiers. At present, the choice is limited to methanol, acetonitrile and tetrahydrofuran (THF). If a good isocratic separation is obtained with any of these three modifiers, the present approach has been succesful and a good chromatogram can be reported. If none of the presently included modifiers yields a good separation, we shall abandon the scheme. In Fig. 1 we have indicated the other figures that we use to make the required decisions. We shall now explain the proposed procedure in detail.

## THEORETICAL

### *Description of isocratic retention behaviour*

As shown previously[4], the retention behaviour of a solute in reversed-phase systems can be expressed by the following linear relationship:

$$\ln k = \ln k_0 - S\varphi \qquad (1)$$

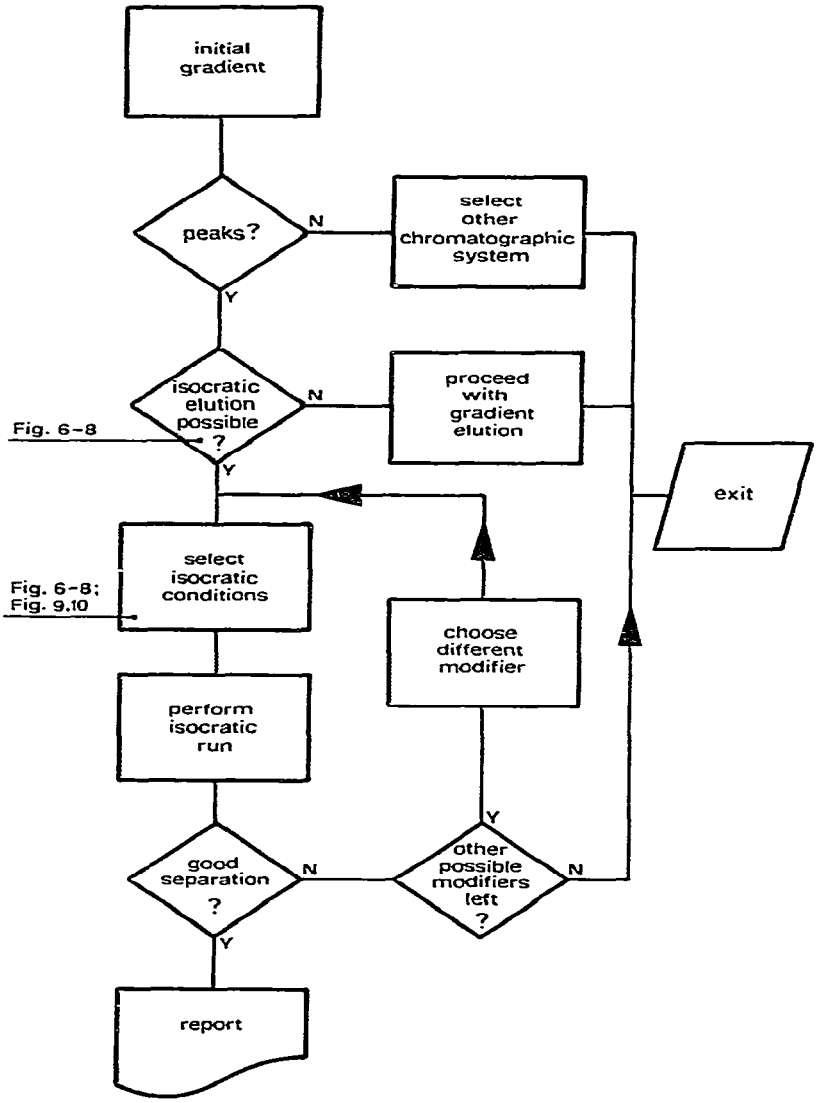



Fig. 1. Schematic presentation of the proposed procedure for the estimation of isocratic elution conditions in RP HPLC.

where $\varphi$ is the volume fraction of the organic modifier. Eqn. 1 is a good approximation of a generally non-linear curve for $k$ values between 1 and 10. As our aim is to derive isocratic conditions yielding such intermediate $k$ values, eqn. 1 is adequate for our purposes. Eqn. 1 shows that two parameters, $\ln k_0$ and $S$, are needed to describe the isocratic retention behaviour. However, we have shown in a previous paper[4] that for some RP systems (notably methanol–water and tetrahydrofuran–water) these two parameters are strongly correlated. This can be expressed as

$$S = p + q \ln k_0 \qquad (2)$$

where $p$ and $q$ are constant for a given binary mobile phase system. Hence, only one parameter ($S$ or ln $k_0$) is needed to describe the isocratic retention behaviour as a function of mobile phase composition for $1 \leqslant k \leqslant 10$.

*Retention behaviour in gradient elution*

Retention behaviour under gradient conditions will be determined by two factors:

(1) the isocratic retention characteristics of the solute (ln $k_0$ and $S$, eqn. 1);

(2) the characteristics of the gradient programme: $\varphi(t)$.

As we noticed before that for methanol–water and, to a lesser extent, for tetrahydrofuran–water there is only *one* parameter needed to describe the isocratic retention behaviour, a knowledge of the characteristics of the gradient programme will permit us to calculate the isocratic retention parameters from *one* experimental data point, *i.e.*, from the retention time in *one* given gradient run.

For linear gradient programmes an exact mathematical relationship between the net retention time under gradient conditions and either of the isocratic retention characteristics ($S$ or ln $k_0$) can be derived. This is done in Appendix I. The results are presented graphically in Figs. 2–4 for linear gradient programmes of interest. Fig. 2

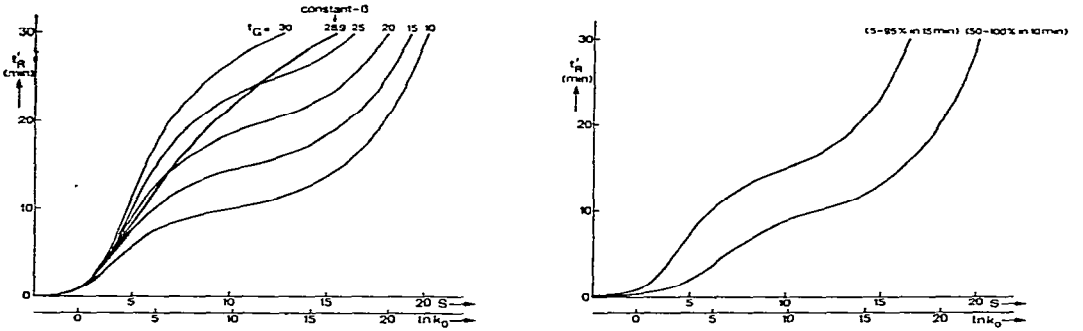



Fig. 2. Relationship between the isocratic retention characteristics $S$ and ln $k_0$ (eqn. 1) and the gradient elution net retention time for a series of linear gradients and for the optimal (constant $\beta$) gradient (eqn. 17). 0–100% methanol–water gradients; $t_0$ = 125 sec.

Fig. 3. Relationship as in Fig. 2 for linear methanol–water gradients running over limited ranges of composition. $t_0$ = 125 sec.

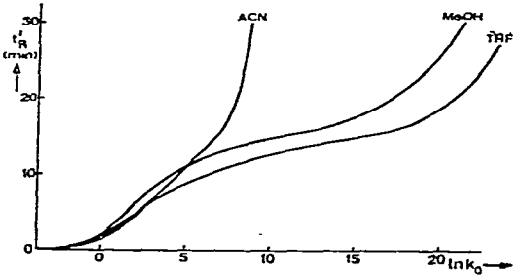



Fig. 4. Relationships as in Fig. 2 for linear gradients of acetonitrile–water and tetrahydrofuran–water. 0–100% linear gradients in 15 min. $t_0$ = 125 sec.

covers a series of linear gradients from 0 to 100% methanol in water with varying gradient steepness. Also included in Fig. 2 is the curve for the so-called constant $\beta$ gradient, which is a convex gradient designed to yield constant elution conditions throughout the chromatogram[4]. Obviously, the constant $\beta$ gradient shows a more regular pattern towards the separation of compounds that differ in $S$ or ln $k_0$ by constant intervals, *e.g.*, homologous series.

Fig. 3 shows the results for linear methanol–water gradients running over limited intervals in composition. The 5–95% gradient might be advisable in practice, as suggested by Dolan *et al.*[2], whereas the 50–100% gradient might be used if very polar compounds are known to be absent from the sample.

Fig. 4 represents 0–100% linear gradients in 15 min of water–acetonitrile and water–tetrahydrofuran mixtures.

It should be noted that the data used to calculate the curves in Figs. 1–3 were derived using the correlation expressed by eqn. 2, which in turn is based on ln $k$ *vs.* $\varphi$ relationships according to eqn. 1 as measured for a set of 32 solutes[4]. Two remarks should be made at this stage:

(1) The parameters in eqn. 1 and hence those in eqn. 2 depend to a certain extent on the value selected for $t_0$, as different $k$ values will be influenced to different extents by changes in $t_0$. In our earlier work we used a $t_0$ value of 107 sec (for a 30 cm × 4.6 mm I.D. column; flow-rate 1.5 ml/min) measured with potassium dichromate[4]. Subsequent experiments have shown that this value underestimates the true hold-up time[5]. Also, the hold-up time might vary with the mobile phase composition[5], but such variations are not allowed in the expressions for gradient elution retention times derived in Appendix I. Therefore, for the present study, we recalculated our experimental data with a constant value for $t_0$ of 125 sec. This value is slightly lower than the smallest retention time observed. Fortunately, it appears that changes in $t_0$ have little influence on the results of our calculations. The parameters describing the three different modifiers used are given in Table I.

TABLE I

GRADIENT SHAPE PARAMETERS DERIVED FROM DATA ON 32 SOLUTES USING A $t_0$ VALUE OF 125 sec (COLUMN POROSITY $\varepsilon = 0.63$)

| *Modifier* | *p* *(eqn. 2)* | *q* *(eqn. 2)* | *r* *(correlation coefficient)* | $t_G$ ($\beta = 0.5$) *(eqn. 17)* |
|---|---|---|---|---|
| Methanol | 2.86 | 0.77 | 0.97 | 28.9 |
| Acetonitrile* | 6.70 | 0 | −0.16 | 27.9 |
| Tetrahydrofuran | 5.62 | 0.68 | 0.65 | 44.0 |

* Because for acetonitrile $S$ and ln $k_0$ (eqn. 1) are not correlated, the corresponding $q$ value should be taken as zero and $p$ represents the average $S$ value for the 32 solutes.

(2) The present approach was based on data from a set of 32 solutes[4]. Although we shall show in this paper that it can be used accurately for solutes not included in this set, we should be careful in extrapolating the scheme, especially towards high-molecular-weight solutes with very high ln $k_0$ values. Such solutes were not included in the original set and might not show the same behaviour.

*$\varphi$ vs. $t'_R$ curves*

Having obtained $S$ from Fig. 2, 3 or 4, we can now proceed to derive the binary solvent composition that will produce a desired isocratic capacity factor. For this purpose we rewrite eqn. 1 as

$$\varphi_k = \frac{\ln k_0 - \ln k}{S} \tag{3}$$

where $\varphi_k$ is the composition at which isocratic elution of the sample component will yield a capacity factor equal to $k$. Substitution of eqn. 2 will now lead to

$$\varphi_k = \frac{S - p - q \ln k}{qS} \tag{4}$$

where $p$ and $q$ are known constants (Table I). Eqn. 4 enables us to calculate the composition at which any $k$ value between 1 and 10 can be expected to occur for a solute with a known $S$ value. We can also calculate the range of binary compositions over which reasonable retention times can be obtained, by estimating the $\varphi_k$ value for $k$ equal to 1 and 10, respectively. The necessary $S$ value may be derived from the retention time obtained from a gradient run (Figs. 2–4). However, it is also possible to integrate eqn. 4 with the data presented in Figs. 2–4 and to plot directly the isocratic binary composition, $\varphi_k$, as a function of the observed gradient retention time, $t'_R$. Consequently, we may derive a curve, $\varphi_1$, relating the retention time of a solute observed in a particular gradient run to the binary composition required to elute it with a value of $k = 1$ under isocratic conditions.

Such figures can be used conveniently to determine proper isocratic conditions. An example is given in Fig. 5, where curves for $\varphi_1$ and $\varphi_{10}$ are presented as a function of $t'_R$, the retention time observed for a 0–100% linear gradient of methanol in water running over 15 min. Here $\varphi_1$ is the curve relating the gradient retention time of a solute with the binary composition required to elute this solute with $k = 1$ under isocratic conditions. Clearly, the less polar solutes, eluting later in the gradient, require a stronger binary eluent (greater $\varphi$). In a similar way, $\varphi_{10}$ presents the results for a desired value of $k = 10$ under isocratic conditions. Now, let us suppose that we

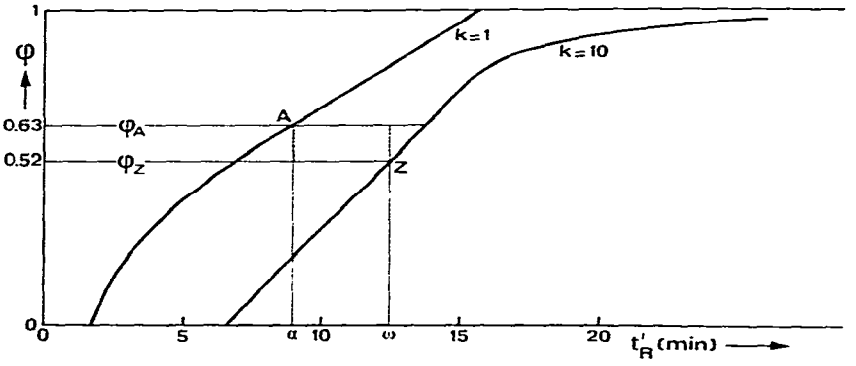



Fig. 5. Schematic illustration of the deduction of proper isocratic conditions from retention data obtained with a 0–100% linear methanol–water gradient in 15 min; $t_0 = 125$ sec.

apply the 0–100% gradient to an unknown sample mixture and that we find sample bands to elute between $t'_R = 9$ and 12.5 min. From Fig. 5 we are now able to predict that under isocratic conditions the first sample band will show a $k$ value equal to 1 for a methanol to water ratio of 0.63:0.37. With decreasing modifier content ($\varphi < 0.63$) the isocratic $k$ value will increase. Conversely, the last sample band has an estimated $k$ value smaller than 10 for an isocratic composition of $\varphi > 0.52$. Hence, for an isocratic binary composition $0.52 > \varphi > 0.63$ all peaks in this particular sample will be eluted between $k = 1$ and 10, and a proper chromatogram can be expected under isocratic conditions.

The graphical procedure is illustrated in Fig. 5. The composition for which the first band is eluted at $k = 1$ is found from the intersection of the vertical at $t'_R = 9$ min with the $k = 1$ curve and is denoted as A. Z indicates the composition where the last peak elutes at $k = 10$. All compositions between A and Z can be expected to yield proper chromatograms. It can be seen from Fig. 5 that the greater the separation between the first and the last peak, (denoted by $\alpha$ and $\omega$), the closer A gets to Z, until finally the value of $\varphi$ at point Z is higher than that of $\varphi$ at point A, and it is no longer possible to elute all bands isocratically with capacity factors between 1 and 10. In this way, Fig. 5 offers a direct criterion for deciding whether a sample can be separated isocratically or should be subjected to gradient elution.

Of course, the condition $1 \leqslant k \leqslant 10$ is arbitrary. If necessary, the permissible range of $k$ could be expanded to, e.g., $0.5 \leqslant k \leqslant 20$, in order to avoid the use of gradient elution or column switching techniques. Hence, curves other than those for $k = 1$ and $k = 10$ could be of importance. In Figs. 6–8 curves have been drawn

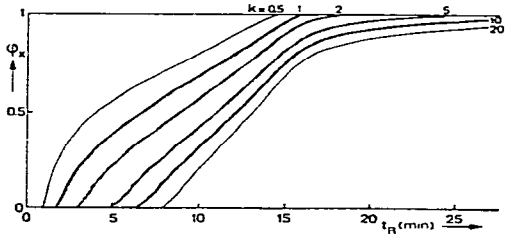
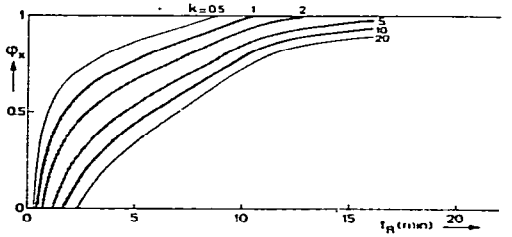



Fig. 6. Curves relating the isocratic composition ($\varphi_x$) to gradient elution net retention time ($t'_R$) calculated for various isocratic capacity factors from eqns. 11 and 12 using data from Fig. 2 and Table I. 0–100% linear methanol–water gradient in 15 min; $t_0 = 125$ sec.



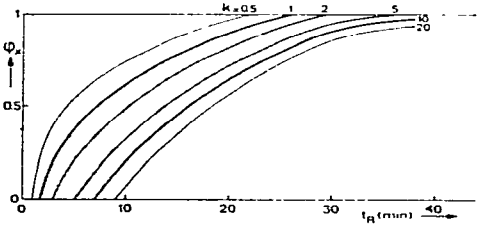




Fig. 7. As Fig. 4, but for a 50–100% linear methanol–water gradient in 10 min, using data from Fig. 3 and Table I.

Fig. 8. As Fig. 4, but for a 0–100% methanol–water constant $\beta$ gradient (Appendix I, with $\beta = 0.5$; $t_G = 28.9$ min).

corresponding to the compositions at which $k$ equals 0.5, 1, 2, 5, 10 and 20, respectively.

All three figures refer to the methanol–water system. Fig. 6 concerns a 0–100% linear gradient ($t_G$ = 15 min), Fig. 7a 50–100% linear gradient ($t_G$ = 10 min) and Fig. 8 the constant $\beta$ (optimal) gradient ($t_G$ = 28.9 min). It should be realized that the proposed procedure relies heavily on the correlation between the solute parameters $k_0$ and $S$, expressed by eqn. 2. It is this correlation which enables us to draw conclusions on the desired isocratic composition from a single gradient retention time. Now, eqn. 2 appears to be most strictly valid in the methanol–water system (*cf.*, Table I). Therefore, we may expect the procedure outlined above to be most accurate if we run our initial gradient with this mobile phase system. For extremely non-polar solutes, where methanol is not a sufficiently strong solvent, we might be forced to use tetrahydrofuran rather than methanol. According to Table I, greater accuracy can be expected for tetrahydrofuran than for acetonitrile–water gradients. However, we should be careful, as extremely non-polar solutes have not been included in the set of 32 that form the basis of Table I. Alternatively, the use of ethanol or propanol might be advantageous for such solutes, because some of our earlier data[6] suggest that eqn. 2 is valid for these modifiers. However, more information is needed for this conclusion to be decisive.

Figs. 6–8 and similar plots offer a very useful guide for the selection of proper isocratic conditions, especially as there is no great influence of the value of $t_0$ on the curves presented. However, if $t_0$ differs greatly from our value of 125 sec, *e.g.*, because short columns are used, it may be advisable to alter the flow-rate. For example, Figs. 6–8 are accurate for 30-cm (4.6 mm I.D.) columns at 1.5 ml/min, and hence also for 20-cm columns at 1 ml/min and for 10-cm columns at 0.5 ml/min. In fact, Figs. 6–8 can be safely used for any value of $t_0$ between roughly 110 and 140 sec with $\varphi_A$ (Fig. 5) much more influenced by the choice of $t_0$ than $\varphi_Z$.

*Transfer from methanol to other organic modifiers*

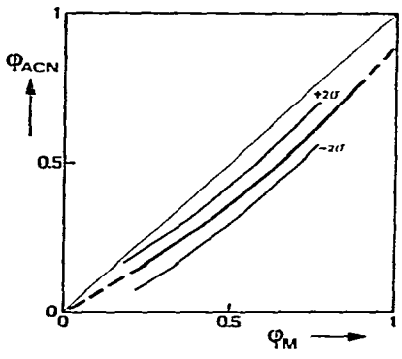
As a result of the above procedure, we obtain a chromatogram where all sample bands are eluted in reasonable time with a certain mixture of (preferably) methanol and water. Whether or not the bands are also sufficiently well separated is another question. In a proper chromatogram, with all $k$ values between 1 and 10, the relative retention of two successive components may vary from 1 (no separation) to 10 (excessive separation). Obviously, slight alterations of the operating parameters, such as mobile phase composition, may improve the selectivity of the system. Simplex optimization has been reported as a possible approach to multi-component separations[7]. Here we shall consider another possibility.

It has been shown that different organic modifiers can produce very significant changes in selectivity[8]. Notably, tetrahydrofuran and acetonitrile show selectivities that can differ considerably from those obtained with methanol. Our earlier data obtained with these three modifiers support this conclusion[4]. Unfortunately, so far we have not been able to predict the changes in selectivity that will occur on replacing methanol by acetonitrile and tetrahydrofuran.

In testing whether it is worthwhile replacing methanol by one of the other modifiers, we want to retain proper isocratic chromatograms in these systems. To obtain such chromatograms, we do not want to repeat the gradient procedure to estimate

the appropriate compositions. Rather, we want to be able to derive these directly from the methanol–water composition estimated from the initial gradient scanning procedure. In other words, we seek a transfer rule that will give us the acetonitrile–water or tetrahydrofuran–water composition that yields the same overall retention as a given methanol–water composition.

Such rules can be derived from the data obtained from the ln $k$ *vs.* $\varphi$ relationship for 32 solutes that were published earlier[4]. We can calculate the average composition of an acetonitrile–water or a tetrahydrofuran–water mixture that will yield the same retention as a given methanol–water mixture. The equations used are derived in Appendix II and the results are given in Figs. 9 and 10.



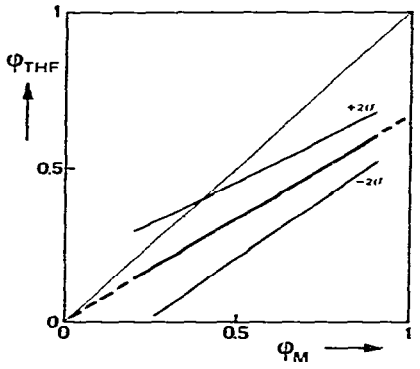




Fig. 9. Transfer curve of equal retention time for the changeover between acetonitrile–water and methanol–water mixtures (eqn. 21). The thick solid curve represents the average calculated from 32 solutes; the thinner curves at $\pm 2\sigma$ indicate variation among the solutes.

Fig. 10. As Fig. 9, but for the changeover between tetrahydrofuran–water and methanol–water mixtures.

Fig. 9 presents the average composition of an acetonitrile–water mixture, $\bar{\varphi}_{ACN}$, as a function of the methanol–water composition ($\varphi_M$). Fig. 10 gives a similar curve for the average tetrahydrofuran–water composition ($\bar{\varphi}_{THF}$). For example, a solute eluting with $k = 2$ in 50% methanol is expected to be eluted with the same $k$ value in 37% acetonitrile or 33% tetrahydrofuran. Note that the $k$ value is not important: it is the equality of the retention times that is essential.

Naturally, the curves in Figs. 9 and 10 represent expected mean data. Individual solutes will deviate from these expectations to an extent indicated by the curves denoted by $+2\sigma$ and $-2\sigma$ in Figs. 9 and 10. These thinner curves mark the 95% confidence interval. To convert this absolute variation in $\varphi$ to a relative variation in $k$, it should be multiplied by the $S$ value, because from eqn. 1

$$\frac{\Delta k}{k} = \Delta \ln k = -S\Delta\varphi \tag{5}$$

Data for $S$ from our previous work[4] show that an error in $\varphi$ of 0.05 can be expected to result in a 25–50% error in $k$. This means that the retention times obtained in acetonitrile–water can differ by up to 50% from those in methanol–water as the uncertainty

in $\bar{\varphi}_{ACN}$ (Fig. 9) is about 0.05. For tetrahydrofuran–water (see Fig. 10) this difference might be up to a factor of 2.

The curve for acetonitrile–water turns out to be significantly non-linear (Fig. 9). If we impose the condition that when $\varphi_M$ is zero $\bar{\varphi}_{ACN}$ should also be zero, we find that the curve for $\bar{\varphi}_{ACN}$ can be accurately described by a second-order polynomial:

$$\bar{\varphi}_{ACN} = 0.32\,\varphi_M^2 + 0.57\,\varphi_M \tag{6}$$

The curve for tetrahydrofuran appears to follow a straight line:

$$\bar{\varphi}_{THF} = 0.66\,\varphi_M \tag{7}$$

With eqns. 6 and 7 we are able to transfer from one organic modifier to another in order to change the selectivity, while keeping the retention approximately constant. This is a very convenient way to try to improve a separation that is insufficient for one modifier. Working along the lines described earlier in this paper, we can transfer an initial isocratic run in methanol–water to one in acetonitrile–water and one in tetrahydrofuran–water in order to find the best possible separation.

## EXPERIMENTAL

All experiments were performed on equipment consisting of one (isocratic) or two (gradient) Model 6000A pumps, a Model U6K injector, a Model 440 UV absorbance detector and a Model 660 solvent programmer, all from Waters Assoc. (Milford, MA, U.S.A.).

One column of length 30 cm and I.D. 4.6 mm was used for all experiments. It was home-packed with Nucleosil 10 C18 (Macherey, Nagel & Co., Düren, G.F.R.).

Methanol and tetrahydrofuran were obtained from Baker (Phillipsburg, NJ, U.S.A.) and acetonitrile from Merck (Darmstadt, G.F.R.). All solvents were of the highest purity available. Water was distilled in our laboratory and treated with ion-exchange resins and carbon filters before use.

Samples were dissolved in mixtures of methanol and water. The PTH-amino acids were prepared in the Laboratory for Organic Chemistry.

During gradient runs, the injection was made 80 sec after the start of the programme, in order to compensate for the delay time[10].

## RESULTS AND DISCUSSION

The theory described above will be elucidated by two illustrative examples.

*Separation of a mixture of phenolic compounds*

A mixture was prepared containing orcinol, phenol, *p*-cresol, 3,4-xylenol, 3,5-xylenol and 2,4-xylenol. To this mixture linear gradients from 0 to 100% methanol in water in 15 min and from 50 to 100% methanol in water in 10 min were applied. The resulting chromatograms are shown in Fig. 11. The relevant peaks are denoted by the numbers 1 to 6 for the phenols in the above sequence. It is obvious that there is a considerable blank signal, caused by impurities in the water. This makes it necessary to

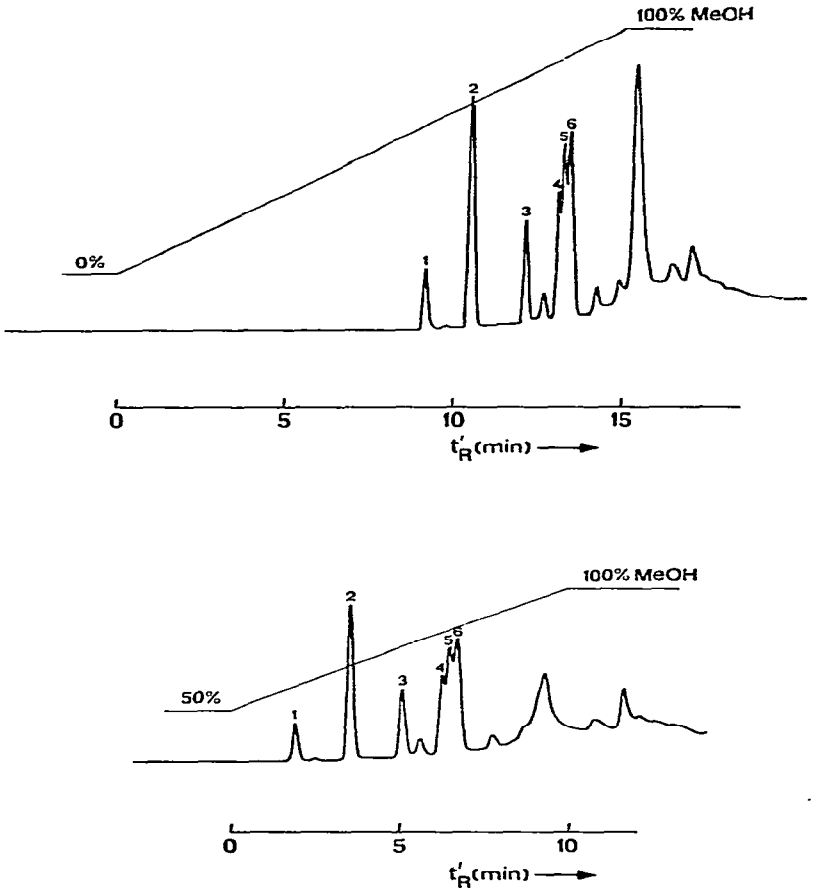



Fig. 11. Gradient elution chromatograms of a mixture of phenolic compounds. Top, 0–100% linear methanol–water gradient in 15 min; bottom, 50–100% linear methanol–water gradient in 10 min. 30 cm × 4.6 mm I.D. column at 1.5 ml/min; UV detection (254 nm). Peak identification: see text.

TABLE II

GRADIENT ELUTION RETENTION TIMES AND RESULTING ISOCRATIC CONDITIONS FOR A MIXTURE OF PHENOLIC COMPOUNDS

| | | |
|---|---|---|
| *Linear methanol–water gradient* | | |
| Composition (%) | 0–100 | 50–100 |
| Gradient time ($t_G$, min) | 15 | 10 |
| *Observed net retention times (Fig. 11)* | | |
| First peak ($\alpha$, min) | 9 | 2 |
| Last peak ($\omega$, min) | 13.5 | 7 |
| *Volume fractions of methanol (Figs. 6 and 7)* | | |
| Maximum ($k = 1$) | 0.63 | 0.58 |
| Minimum ($k = 10$) | 0.59 | 0.59 |
| *Proposed isocratic conditions* | | |
| Methanol–water ($\varphi_M$) | 0.60 | |
| Acetonitrile–water ($\varphi_{ACN}$; Fig. 9) | 0.46 | |
| Tetrahydrofuran–water ($\varphi_{THF}$; Fig. 10) | 0.39 | |

perform a blank, and if the contaminant peaks interfere with the actual chromatogram, further purification of the water is necessary.

The results for the first and last peaks are listed in Table II. The data obtained for the optimal composition range derived from the two gradient runs are in reasonable agreement. The first peak in the 50–100% gradient is eluted quickly ($t'_R$ = 2 min), and it can be seen in Fig. 7 from the steepness of the $k = 1$ curve in this area that the determination of $\varphi_A$ is critical. However, all data in Table I suggest that a mixture of 60% methanol and 40% water should yield a proper chromatogram.

That this is indeed the case is shown in Fig. 12 (top). In the resulting chromato-

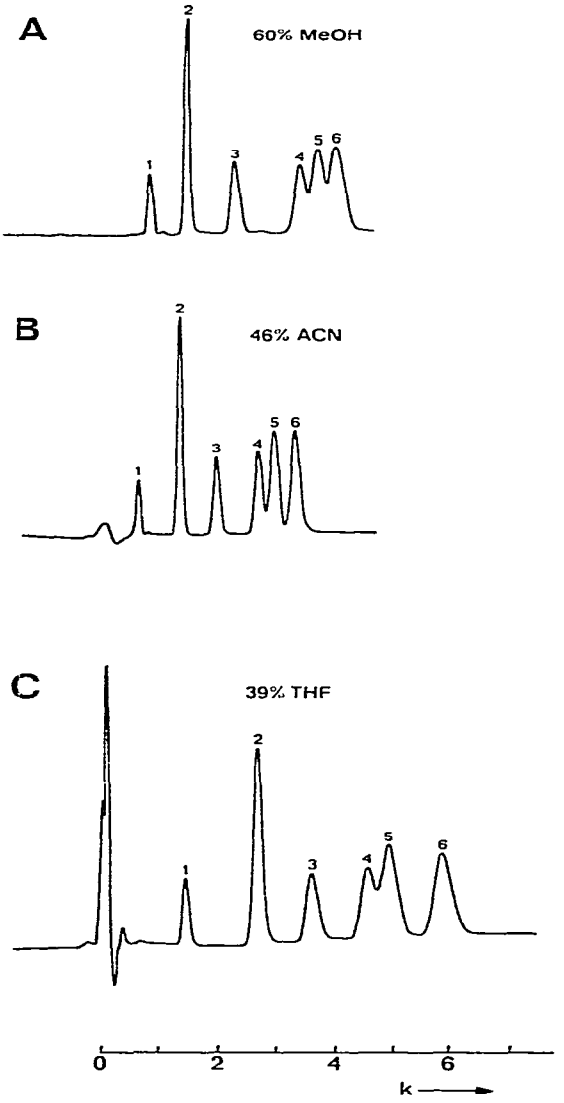



Fig. 12. Isocratic chromatograms of the mixture in Fig. 11 using three different organic modifiers. Compositions obtained from Table II; all other conditions as in Fig. 11. Peaks as in Fig. 11.

gram all peaks are eluted with reasonable retention times. However, the separation is incomplete, especially for the xylenols. Hence, it may be worthwhile to consider different modifiers. From Fig. 9 we find that a $\varphi_M$ value of 0.6 corresponds to a $\bar{\varphi}_{ACN}$ value of 0.46, and from Fig. 10 we find a corresponding $\bar{\varphi}_{THF}$ value of 0.39. The two resulting chromatograms are also shown in Fig. 12 (middle and bottom chromatograms, respectively). Again we obtain proper chromatograms with retention times changing by less than 20% in transferring from methanol to acetonitrile and by 40% for tetrahydrofuran. These values are well within the indicated error margin. A marked improvement of the separation of the xylenols can be observed, especially with acetonitrile–water. Only a slight improvement of the column efficiency is needed to yield baseline separation. The total time needed to obtain this result is about 4 h.

*Separation of some PTH-amino acids*

A mixture of the phenylthiohydantoins of the eight amino acids alanine (Ala), arginine (Arg), glutamic acid (Glu), glycine (Gly), isoleucine (Ile), leucine (Leu), methionine (Met) and phenylalanine (Phe) was subjected to a 0–100% methanol–water linear gradient in 15 min. The result is shown in Fig. 13. Note that the batch of water used was purified and that the contaminant peaks have largely vanished.

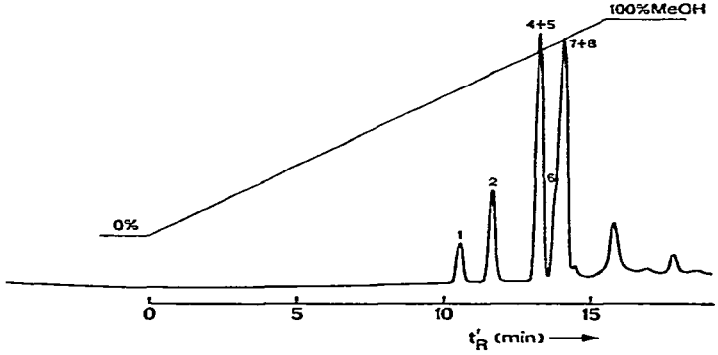



Fig. 13. Gradient elution chromatogram of a mixture of PTH-amino acids. 0–100% linear methanol–water gradient in 15 min; all other conditions as in Fig. 11. Peaks: 1 = Gly; 2 = Ala; 3 = Arg; 4 = Glu; 5 = Met; 6 = Phe; 7 = Ile; 8 = Leu.

Sample peaks are eluted between 10 and about 14 min, leading to an optimal $\varphi_M$ range of 0.69–0.66. From this we expect a proper isocratic chromatogram using 65% methanol. However, although all of the bands in the corresponding chromatogram (Fig. 14a) elute within a reasonable time, their peak shape is poor. Also, the peak of PTH-Arg could not be detected. Therefore, we added 1 m*M* of perfluorooctanoic acid (PFO) to the mobile phase. This is enough to keep the pH of the system slightly acidic, but not enough to promote other possible effects such as ion pairing. The chromatogram in Fig. 14b shows that a considerable increase in efficiency has been achieved without influencing the retention. The PTH-Arg peak is now clearly visible, although it is not very sharp. An increase of the concentration of PFO may improve this peak.

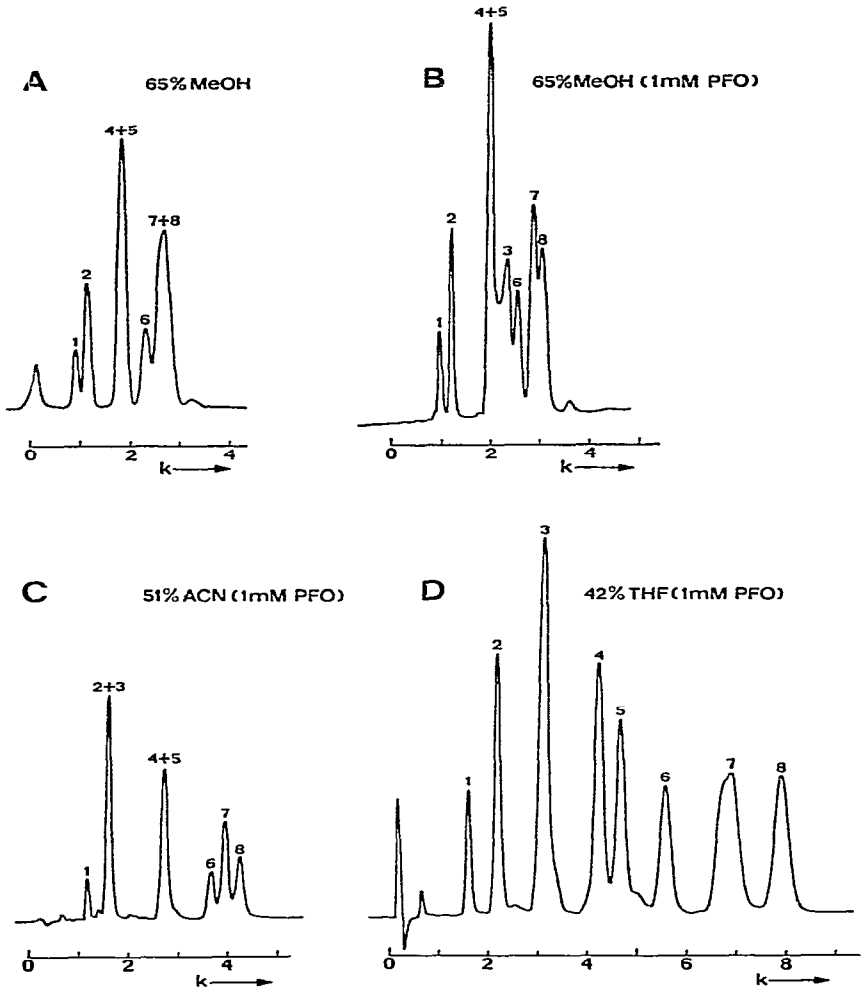



Fig. 14. Isocratic chromatograms of the mixture in Fig. 13 in four different mobile phases. Methanol–water composition obtained from Fig. 6; acetonitrile–water and tetrahydrofuran–water compositions obtained from Figs. 9 and 10 respectively. 1 m*M* of PFO added to the mobile phase where indicated. All other conditions as in Fig. 11. Peaks as in Fig. 13.

Although all peaks are eluted with reasonable retention times, the separation is incomplete. We therefore tried the corresponding mixtures of 51% acetonitrile (see Fig. 9) and 42% tetrahydrofuran (see Fig. 10). The resulting chromatograms are shown in Fig. 14c and d. In both instances 1 m*M* of PFO was added to the mobile phase. It can be seen that two proper chromatograms are obtained, but that the selectivity of the tetrahydrofuran–water system is very much superior. Differences in retention times between the different modifier systems are larger than before, and reach a factor of 2 for tetrahydrofuran compared with methanol.

The present strategy was found to be useful for application to a mixture of compounds all of which are different from the original 32 compounds on which the

scheme was based. Also, the addition of PFO to the mobile phase did not affect this conclusion. Again, about 4 h is sufficient to obtain a good separation.

## CONCLUSIONS

We have shown how gradient elution can be used as a scanning technique for rapidly establishing proper isocratic conditions in RP HPLC. Owing to the observed regularity in the methanol–water system, gradients in this sytem are to be preferred.

Also, once isocratic conditions for the methanol–water system have been established, transfer to acetonitrile–water or tetrahydrofuran–water systems can conveniently be made. In this way the selectivity can be varied, while the retention is kept approximately constant.

The procedure described in this paper offers a highly practical and rapid method for designing isocratic HPLC separations. The method is limited to the reversed-phase system and to detection systems that are compatible with gradient elution. This excludes more or less general detectors such as the refractometer, which is the severest limitation to the application of the proposed strategy.

The examples in this paper show, however, that for the many samples that are compatible with reversed-phase gradient elution HPLC, much time and effort can be saved if the present procedure is applied.

## APPENDIX I

*Relationship between gradient elution retention times and isocratic retention behaviour*

Equations for the retention times of sample components subjected to gradient elution have been derived previously[6]. Exact mathematical solutions for the migration equation can be obtained for linear gradients, assuming a linear or quadratic relationship between $\ln k$ and $\varphi$.

For a linear gradient we can express the change of composition with time as

$$\varphi = a + bt \tag{8}$$

where $a$ is the intitial composition and $b$ the slope of the gradient. If $y$ denotes the final composition, then the time at which the gradient program ends ($t_G$) can be expressed as

$$t_G = \frac{y - a}{b} \tag{9}$$

We have shown before that a linear approximation for the relationship between the logarithm of the isocratic capacity factor and the mobile phase composition is adequate for the description of retention behaviour under gradient conditions. Hence, we assume

$$\ln k(\varphi) = \ln k_0 - S\varphi \tag{10}$$

where $k(\varphi)$ is the isocratic capacity factor as a function of composition.

The following equations are valid for the net retention times of compounds subjected to linear gradients[6]:

(a) If the sample band is eluted before the gradient programme is terminated at the end of the column ($t'_R \leqslant t_G$):

$$t'_R = \frac{1}{S\,b} \ln[1 + S\,b\,t_0\,k(a)] \tag{11}$$

(b) If the sample band is eluted after completion of the gradient ($t'_R \geqslant t_G$):

$$t'_R = k(y)\,t_0 + \frac{1}{S\,b}\left[\frac{k(y)}{k(a)} - 1\right] + t_G \tag{12}$$

In these equations $t_0$ is the dead time of the column, which is assumed to be a constant, *i.e.*, independent of mobile phase composition.

Experimental verification of the above equations has been provided by Hartwick *et al.*[9]. Unfortunately, many commercial HPLC instruments do not deliver gradients that follow eqn. 8[10]. Gradient delay can be accounted for mathematically[6] or by an injection of the sample at the moment at which the gradient arrives at the column inlet. The actual gradient delay time of the system can easily be obtained experimentally[10]. Gradient transformations (deviations from eqn. 6) cannot be dealt with easily. Therefore, we can only assume the HPLC instrumentation to be adequate and the effect to be negligible.

If $S$ and $\ln k_0$ are correlated according to

$$S = p + q \ln k_0 \tag{13}$$

we can substitute eqns. 10 and 13 in eqns. 11 and 12, to obtain

$$t'_R = \frac{1}{S\,b} \ln\left\{1 + S\,b\,t_0 \exp\left[\frac{(1 - aq)S - p}{q}\right]\right\} \tag{14}$$

for $t'_R \leqslant t_G$ and

$$t'_R = t_0 \exp\left[\frac{(1 - qy)S - p}{q}\right] + \frac{1}{S\,b}\left\{\exp[S(a - y)] - 1\right\} + t_G \tag{15}$$

for $t'_R \geqslant t_G$.

For any set of parameters ($t_0$; $a,b,y$; $p,q$), these equations uniquely determine the relationship between $t'_R$ and $S$. Obviously, eqns. 14 and 15 can only be solved numerically towards $S$. However, the reverse calculation of $t'_R$ from $S$ is much more convenient and this naturally points to a graphical procedure for evaluating $S$, *e.g.*, Figs. 2–4.

For non-linear gradients no analytical equations for the net retention times can be derived. We have shown before that, as a consequence of eqn. 13, the optimal shape of a gradient to be applied to a completely unknown sample is

$$\varphi = \frac{1}{q}[1 - (1 - q)^{t/t_G}] \tag{16}$$

In this equation $t_G$ is determined by

$$t_G = \frac{(p + q)\, t_0 \ln(1 - q)}{\beta\, q} \tag{17}$$

where $\beta$ is a constant. $t_G$ represents the gradient time for a 0–100% gradient. For this so-called constant $\beta$ gradient, $t'_R$ values can be obtained by numerical integration[4]. Results for a methanol–water gradient using parameters from Table I are included in Fig. 2.

## APPENDIX II

*Derivation of transfer rules for the substitution of methanol by tetrahydrofuran or acetonitrile*

In each modifier–water system, eqn. 1 can be used to describe the isocratic retention behaviour for $1 \leqslant k \leqslant 10$, *e.g.*, in methanol–water:

$$\ln k^i = \ln k^i_{0,M} - S^i_M \varphi_M \tag{18}$$

where $\ln k^i_{0,M}$ and $S^i_M$ refer to the parameters for compound $i$ in methanol–water. To obtain the same retention in a mixture of acetonitrile and water, the volume fraction of acetonitrile ($\varphi_{ACN}$) should be chosen such that

$$\ln k^i_{0,ACN} - S^i_{ACN} \varphi_{ACN} = \ln k^i_{0,M} - S^i_M \varphi_M \tag{19}$$

or

$$\varphi_{ACN} = \frac{\ln k^i_{0,ACN} - \ln k^i_{0,M} + S^i_M \varphi_M}{S^i_{ACN}} \tag{20}$$

Hence, for each solute, there is a linear relationship between $\varphi_{ACN}$ and $\varphi_M$. In general, different solutes will yield different linear relationships, and hence different values for $\varphi_{ACN}$ for a given value of $\varphi_M$. If we want to transfer from methanol to acetonitrile and keep the retention times roughly constant for a series of unknown compounds, we might use the average $\varphi_{ACN}$ value for a large number of compounds as the best possible estimate. As eqn. 18 is valid over a limited range of capacity factors only, we should limit the averaging at each $\varphi_M$ value to compounds with $1 \leqslant k \leqslant 10$:

$$\bar{\varphi}_{ACN} = \frac{1}{n} \sum_{i=1}^{n} \left| \frac{\ln k^i_{0,ACN} - \ln k^i_{0,M} + S^i_M \varphi_M}{S^i_{ACN}} \right|_{1 \leqslant k \leqslant 10} \tag{21}$$

where $n$ denotes the number of solutes, $i$, for which $1 \leqslant k \leqslant 10$ at $\varphi_M$.

$\bar{\varphi}_{THF}$ can be obtained similarly.

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