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Review

Gradient elution methods for predicting isocratic conditions

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ABSTRACT

Gradient elution methods can elegantly be used for rapidly establishing the appropriate isocratic elution conditions for newly chromatographed samples. In comparison with the conventional approach of running a number of isocratic chromatograms on a trial-and-error basis, gradient elution methods can be much more efficient and yield more consistent results. Key factors are a description of the retention behaviour of the solutes under isocratic conditions as a function of the programmed parameters and an accurate knowledge of the actual gradient profile, *i.e.*, the variation of the programmed parameters with time. Reasonably simple calculation procedures are facilitated by the use of simple (*e.g.*, linear) programmes and instrumentation that affects the actual profile as little as possible. For non-ionic solutes two different gradient scans suffice in principle. In certain cases a single gradient scan may be adequate. A combination of one gradient scan and one isocratic verification experiment can often be used for the accurate prediction of optimum isocratic conditions. For ionic solutes a larger number of scanning experiments are needed than for non-ionic solutes, but in comparison with the time needed for trial-and-error optimization the potential benefits of gradient scanning methods are much greater. For characterizing the ionic solutes in unknown samples two things are needed. First, the solutes need to be classified according to their type (weak or strong; acids or bases) and charge. Next, the optimum isocratic conditions should be established. The concentration of organic modifier, the pH and the type and concentration of ion-pair reagent are the most important parameters to be considered in the process. Using linear gradients at two different pH values in combination with "pulse injection" of ion-pairing reagents, four scanning experiments form the basis of an efficient classification procedure. Once the solutes have been classified, simple, stepwise procedures can be used to establish optimum isocratic conditions.

CONTENTS

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1. INTRODUCTION

Even for compounds of known chemical structure it is usually not possible to predict chromatographic conditions (mobile phase, stationary phase, temperature, pH, etc.) at which elution as sharp peaks in the optimum range of retention times can be achieved. Thus, some experimental data are almost always needed to establish appropriate conditions for elution. One way to elute a wide variety of samples under approximately optimum conditions in high-performance liquid chromatography (HPLC) is to apply solvent programming or gradient elution techniques. This implies that the mobile phase is somehow varied during the chromatographic run. Solvent programming may involve stepwise ("step gradients") or continuous (linear, convex or concave gradients) variations in the concentration of solvent components, the pH or the ionic strength. Combinations (multi-segment gradients) are also possible. Varying the stationary phase is not practical in LC, but temperature programming is. The latter technique, however, is much less powerful in HPLC than it is in gas chromatography, because the retention of solutes cannot be varied by several orders of magnitude within the practical temperature range. Such large variations can be realized with the most widely applied gradient elution methods, *i.e.*, the programming of the mobile phase composition (or "solvent strength") for neutral solutes, ionic strength for ionic solutes and pH for ionogenic solutes. Aspects of these various gradient elution methods will be considered in this paper.

Gradient elution methods may be used in several different ways:

(1) for the elution of samples of which the individual components require vastly different elution conditions;

(2) for scanning unknown samples for the presence of a wide variety of components; and

(3) for predicting suitable non-programmed (*i.e.*, isocratic, isoprotic, etc.) elution conditions for unknown samples.

The purpose of this paper is to review the application of gradient elution techniques for the third purpose. Several advantages of gradient elution methods can be identified:

(i) potentially, all components of the sample can be eluted in a single experiment, including unexpected contaminants;

(ii) no *a priori* information on suitable non-programmed elution conditions is needed; and

(iii) approximately constant resolving power and sensitivity can be maintained throughout the run.

These advantages need to be balanced against the following disadvantages:

(i) the detection techniques used need to be compatible with gradient elution methods and thus need to be selective; as a consequence, certain sample components may be overlooked;

(ii) the accuracy of the predictions is limited by experimental errors and non-idealities, assumptions regarding the (approximate) behaviour of solutes and imprecise calculation procedures.

1.1. Optimum isocratic elution range

If it is our aim to predict conditions under which retention times in the optimum range can be obtained, then this optimum range should be defined. For separating two compounds, the optimum capacity factor can be found from the equation describing resolution ($R_{s,ji}$) as a function of retention (the average capacity factor $\bar{k} = \frac{1}{2}k_i + \frac{1}{2}k_j$), selectivity ($\alpha_{ji} = k_j/k_i$) and efficiency (plate count, N) [1]:

$$R_{s,ji} = \left(\frac{\bar{k}}{1+\bar{k}}\right)\left(\frac{\alpha_{ji}-1}{\alpha_{ji}+1}\right)\frac{\sqrt{N}}{2} \tag{1}$$

The efficiency can be related to the column length (L) and the height equivalent to one plate (H) by $N = L/H$. The column length is the product of the linear velocity (u) and the time (t_0) needed for an unretained solute to pass through the column ($L = ut_0$). The average time needed for solutes i and j is $\bar{t} = \frac{1}{2}t_i + \frac{1}{2}t_j = t_0(1 + \bar{k})$. Substitution of these relationships in eqn. 1 yields an expression for the required (average) retention time of the two solutes:

$$\bar{t} = 4R_{s,ji}^2\left[\frac{(1+\bar{k})^3}{\bar{k}^2}\right]\left[\frac{(\alpha_{ji}+1)^2}{(\alpha_{ji}-1)^2}\right]\frac{H}{u} \tag{2}$$

From the derivative of $\bar{t}$ with respect to $\bar{k}$, we find that two peaks can be separated most rapidly if the average capacity factor equals 2. The capacity factor of the first peak can be found from

$$k_1 = \bar{k} - \frac{2R_s(\bar{k}+1)}{\sqrt{N}} = 2 - \frac{6R_s}{\sqrt{N}} \tag{3}$$

whereas for the second peak

$$k_2 = \bar{k} + \frac{2R_s(\bar{k}+1)}{\sqrt{N}} = 2 + \frac{6R_s}{\sqrt{N}} \tag{4}$$

For separating more than two peaks a broader range of capacity factors will need to be covered. For three peaks we may calculate the optimum values, assuming that the resolution between the two pairs of peaks is the same (*i.e.*, $R_{s,32} = R_{s,21}$) and that the values of the function $(1 + \bar{k})^3/\bar{k}^2$ are equally far from the optimum value of 6.75 for each pair of peaks. These criteria are met if $k_2 = 1 + \sqrt{1 - 4R_s/N}$. This means that

the centre peak will remain located very close to the "optimum value" of 2. The capacity factor of the first peak can be found from

$$k_{j-1} = \frac{k_j(\sqrt{N} - 2R_s) - 4R_s}{\sqrt{N} + 2R_s} \tag{5}$$

whereas for the last peak

$$k_{j+1} = \frac{k_j(\sqrt{N} + 2R_s) + 4R_s}{\sqrt{N} - 2R_s} \tag{6}$$

Using eqns. 5 and 6 the positions of series of peaks can be calculated assuming the middle peak [number $\frac{1}{2}(n + 1)$ in the case of n peaks] to be eluted at $k = 2$. In the case of an even number of peaks the average k value of the two peaks in the middle (numbers $\frac{1}{2}n$ and $\frac{1}{2}n + 1$) can be taken to equal 2. The resulting peak positions can be used to establish suitable capacity factor ranges for multi-component mixtures. Values under two different sets of conditions, *i.e.*, $R_s = 2$, $N = 10^4$ and $R_s = 5$, $N = 5000$, are listed in Table 1.

Obviously, more peaks can be separated in a certain interval if the mutual distances become smaller (*e.g.*, $R_s = 2$ instead of $R_s = 5$). In the example in Table 1 in which the peaks are more widely spread, it appears not to be possible to elute more than eight peaks in the range $1 < k < 20$ with the middle peak(s) around $k = 2$.

Allowing capacity factors all the way down to zero is usually not advisable. Some system peaks or just baseline disturbances are always likely to be present close to $k = 0$, and the presence of solvent peaks may force us to aim to elute all relevant peaks with capacity factors above a certain value (*e.g.*, $k = 0.5$ or $k = 1$). If the k value for the first peak suggested by Table 1 (or by the repeated use of eqn. 5) falls below this threshold, then the following equation may be used to establish a desirable range of k values:

$$k_\omega = (1 + k_\alpha) \exp\left[\frac{4R_s(n-1)}{\sqrt{N}}\right] - 1 \tag{7}$$

where k_ω is the capacity factor of the last peak, k_α the (desired) capacity factor of the first peak and n the number of peaks. This equation may, for example, be used to establish that ten peaks can be eluted with $R_s = 5$ and $N = 10^4$ between $k_\alpha = 1$ and $k_\omega \approx 11$.

Both in Table 1 and in eqn. 7, it has been assumed that all peaks are distributed evenly thoughout the chromatogram. Of course, this is unlikely to be the case for "new" samples, *i.e.*, early in the development of chromatographic methods. Assuming that the peaks are distributed randomly, the required peak capacity (n_p) for resolving n peaks can be estimated [2]. In this case, Table 1 and eqn. 7 can be used by using n_p instead of the actual number of peaks n.

Often, once the components in a sample have been eluted within the optimum range of k values, the selectivity of the separation will subsequently be optimized. If this is the case, the assumption of a random distribution of the peaks becomes

TABLE 1

OPTIMUM CAPACITY FACTORS FOR SEPARATING GIVEN NUMBERS OF PEAKS

Data calculated using eqns. 5 and 6, assuming the middle peak to be located at $k = 2$ for odd numbers of peaks or around $\bar{k} = 2$ for even numbers of peaks. Values of k above 20 (and below 0) are not given.

Odd numbers of peaks			Even numbers of peaks		
No. of peaks	$R_s = 2$, $N = 10^4$	$R_s = 5$, $N = 5000$	No. of peaks	$R_s = 2$, $N = 10^4$	$R_s = 5$, $N = 5000$
21	0.35	–	20	0.40	–
19	0.46	–	18	0.52	–
17	0.58	–	16	0.64	–
15	0.71	–	14	0.78	–
13	0.86	–	12	0.93	–
11	1.01	–	10	1.09	–
9	1.18	–	8	1.27	0.10
7	1.36	0.28	6	1.45	0.46
5	1.56	0.70	4	1.66	0.94
3	1.77	1.26	2	1.88	1.58
1	2.00	2.00			
3	2.25	2.99	2	2.12	2.42
5	2.52	4.30	4	2.38	3.55
7	2.81	6.05	6	2.66	5.05
9	3.13	8.37	8	2.97	7.05
11	3.48	11.46	10	3.30	9.70
13	3.85	15.56	12	3.66	13.22
15	4.25	–	14	4.04	17.90
17	4.69	–	16	4.46	–
19	5.17	–	18	4.92	–
21	?	–	20	5.41	–

unrealistic. A typical series of actions in developing HPLC separations is to perform retention optimization, selectivity optimization and system optimization. In modern software for system optimization [3,4] the required resolution can be specified. This required resolution refers to the lowest value for R_s between any two peaks of which at least one is relevant. All other (relevant) values for R_s in the chromatogram will be higher. Therefore, the required range of capacity factors will be broader than suggested by Table 1 or eqn. 7. The required value for N depends very much on the results of the selectivity optimization. However, if an HPLC separation is successfully developed, the required plate count should not exceed *ca.* 10 000. A reasonable practical estimate for the required capacity factor range may be obtained by using twice the value for R_s that will be specified during the system optimization stage, a reasonable value for the required number of plates (*e.g.*, $N = 10^4$) and eqns. 5–7 or Table 1. An optimization process in which the required capacity factor range is adapted based on the results obtained during the selectivity optimization stage may seem attractive, but one should be aware that most methods used to affect the capacity factors will also have an effect on the selectivity.

1.2. Principles of gradient scanning methods

The use of gradient elution techniques for predicting isocratic elution conditions is based on the ability to describe the retention of solutes under gradient conditions from known data on their isocratic behaviour [5–8] and, most important for the present application, *vice versa*. Let us assume that the capacity factor $k(x)$ is some function of the parameter x, which is programmed during the chromatographic run. The gradient elution programme is described by the function f:

$$x = \mathrm{f}(t) \tag{8}$$

A gradient programme will need a certain time (the so-called delay time, τ) to reach the top of the column and a further time z/u, with u being the linear velocity of the mobile phase ($u = L/t_0$), to reach a certain point z in the column. Assuming that the function f itself is not affected by the process (*i.e.*, no significant deformations occur due to the instrumentation and connections, or due to selective retention of solvent components on the column; see section 1.3), we find

$$x(z,t) = \mathrm{f}\left(t - \frac{z}{u} - \tau\right) \tag{9}$$

and, after introducing the inverse function f^{-1},

$$t = \frac{z}{u} + \tau + \mathrm{f}^{-1}(x) \tag{10}$$

or

$$\mathrm{d}t = \frac{\mathrm{d}z}{u} + \mathrm{df}^{-1}(x) \tag{11}$$

The migration velocity of the solute is given by

$$\frac{\mathrm{d}z}{\mathrm{d}t} = \frac{u}{1 + k(x)} \tag{12}$$

Eliminating $\mathrm{d}t$ from eqns. 11 and 12 yields

$$\frac{\mathrm{df}^{-1}(x)}{k(x)} = \frac{\mathrm{d}z}{u} \tag{13}$$

Eqn. 13 can be integrated if we realize that z varies between 0 and the column length L and the inverse function f^{-1} (see eqn. 10) varies between a value of $-\tau$ at $t = 0$ and $t_R - t_0 - \tau = t'_R - \tau$ when the solute elutes from the column ($z = L$). Until the solute is overtaken by the gradient programme at the time $t = \tau + z/u$ (or $\mathrm{f}^{-1} = 0$), the programming parameter will maintain its initial value x_0 and the capacity factor will likewise remain constant [$k = k(x_0)$]. We now find

$$\int_{-\tau}^{0} \frac{\mathrm{df}^{-1}(x)}{k(x_0)} + \int_{0}^{t'_R-\tau} \frac{\mathrm{df}^{-1}(x)}{k(x)} = \int_{0}^{L} \frac{\mathrm{d}z}{u} = t_0 \tag{14}$$

or

$$\int_{0}^{t'_R-\tau} \frac{\mathrm{df}^{-1}(x)}{k(x)} = t_0 - \frac{\tau}{k(x_0)} \tag{15}$$

The two vital ingredients of eqn. 15 are the function f describing the gradient programme and the relationship $k(x)$ between the capacity factor and the programmed parameter. Once these two functions are known, the integral equation can always be solved numerically to yield a value for t'_R. In a number of instances analytical solutions can be found. For a summary of these, the reader is referred to the literature [7]. Here we shall just consider one particularly important example, namely that of a linear composition gradient in reversed-phase liquid chromatography (RPLC). Over a limited range of composition, the function $k(\varphi)$ that describes the variation of the solute capacity factor with the volume fraction φ of the organic modifier in the mobile phase can be approximated by

$$\ln k(\varphi) = \ln k_0 - S\varphi \tag{16}$$

where k_0 is the extrapolated capacity factor in pure water and S is the slope of the logarithmic plot. A linear gradient can be described by

$$\varphi = a + bt \tag{17}$$

so that the inverse function becomes

$$\mathrm{f}^{-1}(\varphi) = \frac{\varphi - a}{b} \tag{18}$$

Substitution of eqns. 16 and 18 into eqn. 15 yields

$$\int_{0}^{t'_R-\tau} \frac{\mathrm{d}\,\frac{\varphi - a}{b}}{k_0 \mathrm{e}^{(-S\varphi)}} = t_0 - \frac{\tau}{k(a)} \tag{19}$$

or

$$\frac{1}{bk_0} \int_{a}^{a+b(t'_R-\tau)} \mathrm{e}^{S\varphi}\,\mathrm{d}\varphi = t_0 - \frac{\tau}{k(a)} \tag{20}$$

which yields

$$t'_R = \frac{1}{Sb} \ln \left\{ 1 + Sbk(a) \left[t_0 - \frac{\tau}{k(a)} \right] \right\} + \tau \tag{21}$$

If the solute is eluted after completion of the gradient, *i.e.*, after the final composition y has been reached at the end of the column [$t'_R \geqslant (y - a)/b + \tau$], then

$$t'_R = k(y) \left[t_0 - \frac{\tau}{k(a)} \right] + \frac{k(y)}{Sb} [k(y) - k(a)] - \frac{a - y}{b} + \tau \tag{22}$$

These equations can be simplified if the sample is injected at the time at which the gradient programme reaches the top of the column, *i.e.*, at $t = \tau$. In that case we find

$$t'_R = \frac{1}{Sb} \ln [1 + Sbt_0k(a)] \tag{23}$$

and

$$t'_R = k(y)t_0 + \frac{k(y)}{Sb} [k(y) - k(a)] - \frac{a - y}{b} \tag{24}$$

for elution prior to and after completion of the gradient, respectively.

1.2.1. Ionogenic solutes. With the exception of ion-exchange chromatography [10], the description of gradient elution experiments using eqn. 15 is more difficult for ionogenic solutes. This is due to both experimental [controlling the inverse gradient function $f^{-1}(x)$] and fundamental [the function $k(x)$] difficulties. In many cases gradients are used to obtain qualitative (charge, type) rather than quantitative information.

Based on theoretical considerations from an equilibrium retention model, Foley and May [11] suggested that pH gradients in the reversed-phase mode could potentially be useful for the separation of multi-component mixtures of weak acids or weak bases. The problem of running linear gradients over a broad pH range with good buffer capacity was satisfactorily tackled [12,13]. Linear gradients from pH 3 to 9 were generated by using two pumps and mixing a 0.05 *M* "universal buffer" (consisting of acetic, phosphoric and boric acids) and a 0.1 *M* sodium hydroxide solution. The flow-rate of the two pumps had to be controlled by a special computer programme [13], which is not readily available for others. The difficulty of realizing linear or otherwise well characterized pH gradients may be the main reason why the usefulness of pH gradients for predicting suitable initial conditions for optimization procedures has not been evaluated.

In contrast, organic modifier gradients at constant eluent pH have been found extremely useful for classifying the components of more or less unknown samples according to their charge and "type" (see section 3.2.). In this context, the type of a solute refers to whether it is neutral, strongly or weakly acidic, or strongly or weakly basic.

The use of micellar mobile phases for gradient elution in reversed-phase

chromatography has been demonstrated. Micellar gradients, in which the concentration of micelles increases with time, can be used to elute sample mixtures covering a broad polarity range [14] and are compatible with electrochemical detection [15]. In principle, micellar gradients may be used to determine initial mobile phase conditions prior to the optimization of micellar chromatographic separations.

1.3. Potential complications

From eqn. 14 it is apparent that there are two fundamental sources of error in predicting retention times under gradient conditions: errors in the inverse gradient function $f^{-1}(x)$ and errors in the relationship between the capacity factor and the programmed parameter $k(x)$. Errors in the gradient function generally reflect differences between the desired gradient programme $x(t)$, as entered by the user, and the actual variation $x_{obs}(t)$ of the programmed parameter with time [16,17]. One such variation is the gradient delay time τ, which has been accounted for in the mathematics in the previous section. However, there it was assumed that the function $x(t)$ was otherwise left unchanged. The programmed function is affected by instrumental factors: mixing volumes, inaccurate or imprecise mixing, pump efficiency, etc. Generally, the actual gradient function will therefore differ from the programmed function. If the deviations are (quantitatively) known, they can be accounted for mathematically [16]. However, in practical situations this is often not the case. Steeper gradients, lower flow-rates and higher pressures tend to cause greater differences between $x(t)$ and $x_{obs}(t)$. It is important to select instrumentation that is as good as possible for the purpose, *i.e.*, that shows the smallest possible differences between the desired and the actual gradients. Certain pieces of instrumentation, such as sample loops with very large internal volumes, are not recommended for use in the present application.

Another source of variations is the deformation of the gradient programme by the column itself [18], most importantly because of preferential adsorption of solvent components by the stationary phase, an effect sometimes referred to as "solvent demixing". In some instances, *e.g.*, in ion-pair chromatography, this effect can be so large as to impair the practical application of gradient elution techniques. In many other instances it is a significant factor.

The second major category of complications is formed by variations in the relationship between k and x. Only a reasonable description of this relationship is needed to predict retention times under gradient conditions [9,18]. This implies that the reverse process, obtaining information on the $k(x)$ relationship from gradient elution retention data, will be sensitive to errors. Assumptions made about the relationship between k and x and about the parameters involved, for example assuming retention in RPLC to vary with composition according to $\ln k = \ln k_0 - S\varphi$ with $S = p + q \ln k_0$ (see section 2), can significantly affect the outcome of calculation procedures.

The accuracy of prediction methods based on gradient elution will be considered in the relevant sections below.

1.4. Alternative scanning methods

At the beginning of this paper the advantages and disadvantages of gradient scanning techniques were summarized. The main disadvantages, limited accuracy and restricted detection possibilities, could possibly be overcome by using other scanning

techniques. One obvious improvement is to follow the gradient experiment by the (predicted) non-programmed run and to improve the conditions if necessary. In such a second experiment both of the major disadvantages of gradient scanning methods disappear and therefore this method is to be preferred over the use of two different gradient elution experiments.

Instead of a single gradient experiment, a number of isocratic runs may be used to establish optimum retention conditions [19]. The obvious disadvantage of this method is the number of experiments required. It is important to do a first experiment with a strong eluent, *e.g.*, 100% organic modifier in RPLC, so that relevant solutes will not adhere to the column. The solvent strength can then be lowered in a carefully considered manner, *e.g.*, by using large steps first, followed by "fine tuning" of the eluent.

If thin-layer chromatography (TLC) is used as a scanning technique, the risk of non-migrating solutes is eliminated. Therefore, an experiment on a TLC plate in support of a column experiment is often worth considering. However, TLC is much more attractive as a method for finding an appropriate stationary phase than for establishing the optimum mobile phase composition. In the latter instance the technique is much more time consuming and much less easily automated than column LC.

A final way to predict conditions under which retention times in the optimum range can be achieved is to make use of existing data on known (related) compounds and to predict the behaviour of unknown (*e.g.*, newly synthesized) solutes. Many studies on so-called "quantitative structure–retention relationships" have been performed. In all instances, vast amounts of data are needed on very closely related compounds. A potential way to improve this situation is by the use of expert systems. These allow heuristic knowledge and experience from a chromatographer to be captured in a computer programme. In one recent system optimum conditions are predicted for basic drugs. The predictions are verified experimentally and subsequently improved [20].

2. NEUTRAL SOLUTES

2.1. Two gradient scans

Assuming that the variation of retention with composition can be described by eqn. 16, we need to determine two coefficients, $\ln k_0$ and S, to characterize the behaviour of a solute. In principle, we need two data points to determine the two unknown coefficients. More generally, this will be the case in any situation in which two parameters define the relationship between retention under non-programmed conditions and the programmed parameter, in other words, in any situation in which a straight line can be obtained by plotting some known function of the capacity factor *vs.* some known function of the programmed parameter. In applying two different gradients, the analyst can be reasonably assured that all detectable compounds actually appear in the chromatogram. Thus, running two different gradient programmes is an effective way to obtain the two required data points. This strategy is, for example, applied in the DryLab programme developed by Snyder *et al.* [21,22].

When the solutes elute before the completion of the gradient, and assuming for simplicity that the solutes are injected after a delay time τ, *i.e.*, when the gradient

actually reaches the top of the column, we may write for the retention times of a solute i subjected to two different linear gradients

$$t'_{R_{i,1}} = \frac{1}{S_i b_1} \ln [1 + S_i b_1 t_0 k_i(a_1)] = \frac{1}{S_i b_1} \ln (1 + S_i b_1 t_0 k_{0,i} e^{-S_i a_1}) \quad (25)$$

and

$$t'_{R_{i,2}} = \frac{1}{S_i b_2} \ln [1 + S_i b_2 t_0 k_i(a_2)] = \frac{1}{S_i b_2} \ln (1 + S_i b_2 t_0 k_{0,i} e^{-S_i a_2}) \quad (26)$$

Knowing the starting conditions (a_1 and a_2, respectively) and the slopes (b_1 and b_2) of the two gradient programmes, the coefficients k_0 and S can be established using eqns. 25 and 26. The more different the slopes of the gradients (b_1 and b_2) are chosen, the more precise will be the estimate for S [23]. There is no analytical solution to the set of two equations, but when using a computer numerical methods are rapid and convenient [23].

While the use of two gradients may potentially provide more accurate estimates of the parameters describing the isocratic retention behaviour of a solute, there are also some additional problems [24]. The most significant of these is the need to match correctly the peaks of the individual solutes in the two chromatograms. Reversals in peak orders form a complication for methods based on two gradient scans. However, when single-scan methods are used the same effect causes inaccurate predictions.

2.2. Single gradient scan

In the previous section we investigated the possibility of characterizing the isocratic retention behaviour of solutes based on two gradient runs. If only one parameter is needed to characterize the isocratic elution behaviour of a solute, then a single gradient would suffice. Suggestions have been made for "rules of thumb" that provide an estimate of suitable isocratic elution conditions based on a single gradient scan. For example, Snyder *et al.* [5] suggested that for a component eluting after a time t_R under gradient conditions, the composition at the column inlet at a time $t_R - 2.5t_0$ would be a good starting point for isocratic elution. However, it is a prerequisite for successful gradient elution experiments that the programmed parameter has a strong effect on retention, and at least two parameters (and thus two experimental data points) are normally needed to characterize such an effect. One experimental data point (*i.e.*, one gradient scan) would only suffice if there is some known relationship between the two parameters (slope and intercept). The most significant example of a situation in which this is the case occurs in RPLC when using methanol (and to some extent THF) as the organic modifier [25].

The theoretical basis for the rapid determination of suitable isocratic conditions from a single linear gradient in RPLC originates from a study of more than 50 different solutes eluted under isocratic conditions from an ODS stationary phase using binary methanol–water mixtures [9]. Over a limited range ($1 < k < 10$) the retention behaviour of a solute in such systems could be accurately described by eqn. 16.

Moreover, the slope S and the intercept $\ln k_0$ were shown to be highly correlated. The equation

$$S = p + q \ln k_0 \tag{27}$$

has been found to be approximately valid, at least for well defined groups of solutes. The values obtained for the parameters in ref. 9 were $p = 2.86$ and $q = 0.77$. The generality of these coefficients has been disputed by Berridge [26], who argued that p and q were likely to depend on the type of the column, in addition to the hold-up time (t_0) and the flow-rate. The stationary phase must doubtlessly have some effect, but k values (and thus p and q values, which are derived from these) are principally independent of the flow-rate. When a consistent method is used to determine or estimate t_0, errors in its value should not greatly affect k values, at least not for capacity factors larger than 1 (eqn. 16 is assumed to be valid only over the range $1 < k < 10$).

Hafkenscheid and Tomlinson [27] compared values of p and q from four different sources, obtained from large numbers of data on three different columns (Hypersil-ODS, Nucleosil 10RP-18, and LiChrosorb RP-18). They found good agreement between the different values for p and (especially) q and proposed to use the average values to obtain the most reliable estimates for S. The suggested values were where $p = 3.592$ and $q = 0.74$. Calculations based on both sets of parameters (*i.e.*, those of Schoenmakers *et al.* [25] and those of Hafkenscheid and Tomlinson [27]) show a maximum difference in the predicted optimum mobile phase composition of 2.5% (see Table 2). This illustrates that in applying eqn. 27 for gradient scanning purposes the values of p and q are not a major source of error.

If eqn. 27 holds, eqn. 23 (again, for reasons of simplicity, assuming the sample to be injected at $t = \tau$) can be written explicitely in terms of either k_0 or S. The latter, simpler, equation is

TABLE 2

PREDICTED ISOCRATIC METHANOL–WATER BINARY ELUENT COMPOSITIONS (φ) AND CAPACITY FACTORS FOR THE FIRST PEAK (k_α) ON THE BASIS OF HYPOTHETICAL GRADIENT DATA

t'_α = net retention time of first peak under gradient conditions; t'_ω = gradient elution retention time of last peak. Gradient programme, 0–100% methanol in water in 15 min; $t_0 = 2$ min. Isocratic capacity factor for the last peak, $k_\omega = 10$. Coefficients in eqn. 27 were taken from Schoenmakers *et al.* [25] ($p = 2.86$, $q = 0.77$) and from Hafkenscheid and Tomlinson [27] ($p = 3.592$, $q = 0.74$).

t'_α (min)	t'_ω (min)	Ref. 25		Ref. 27	
		φ	k_α	φ	k_α
4	6	0.044	3.9	0.067	3.6
4	8	0.217	1.9	0.232	1.7
4	10	0.371	1.0	0.383	0.8
4	12	0.521	0.6	0.531	0.4
4	14	0.673	0.3	0.683	0.2
4	16	0.833	0.2	0.841	<0.1

$$t'_{R_i} = \frac{1}{S_i b} \ln \left[1 + S_i b t_0 e^{\frac{S(1-qa)-p}{q}} \right] \quad (28)$$

Although eqn. 28 cannot be made explicit in S, it can easily be solved graphically or numerically [25]. In this respect, an important characteristic of the equation is that it is monotonous, *i.e.*, t'_{R_i} always increases with increasing S.

Fig. 1 can be used to estimate graphically the optimum binary composition of a methanol–water mixture based on the net retention times of solutes under standard gradient conditions (0–100% linear gradient in 15 min; $t_0 = 125$ s). Fig. 1 incorporates eqns. 16, 27 and 28. Non-ideal processes (instrumental and chromatographic) can reduce the accuracy of the above equations and thereby lead to errors in the estimated binary composition. Deviations of eqn. 16 or 27 are specific to the physical and chemical properties of the solute. Instrumental and experimental problems, such as malfunctioning of equipment, should be taken care of by the operator instead of being included in the procedure. The gradient delay time can be taken into account mathematically, but in drawing Fig. 1 it has been assumed that $\tau = 0$, which corresponds to injection of the sample a time τ after starting the gradient programme.

The desired capacity factor range ($k_\alpha^* \leqslant k \leqslant k_\omega^*$, where k_α^* is the minimum desirable capacity factor for the first peak and k_ω^* the maximum desirable capacity factor for the last peak) can be taken into account in a gradient scanning procedure. De Galan *et al.* [28] calculated the peak capacity from

$$N_c = \frac{\sqrt{N}}{4R_{s,req}} \ln \left(\frac{1 + k_\alpha}{1 + k_\omega} \right) + 1 \quad (29)$$

where N_c is the peak capacity, $R_{s,req}$ is the required resolution and N is the plate count. The statistical approach developed by Herman *et al.* [29] can be used to determine the peak capacities needed to achieve a separation with a given probability of success. This can be used to determine which capacity factor range should be aimed for. The following data are needed: how many solutes are present, how many of these are of analytical interest, what is the polarity range of the sample, what is the actual plate

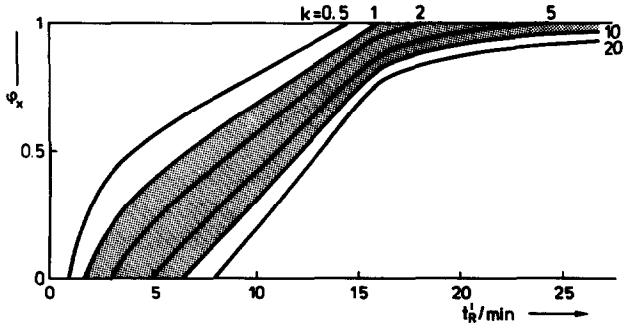

Fig. 1. Graphical presentation of the relationship between gradient elution (net) retention times and isocratic composition. Lines represent the required composition to achieve the indicated capacity factor. Linear gradient from 0 to 100% of methanol in water, $t_0 \approx 2$ min. Column, ODS. Reprinted from ref. 25 with permission.

count of the column and what resolution will be required for the peaks of interest? In this procedure, interpreting the result of a gradient scan becomes very important. The total number of components in the mixture, the number of components which are of analytical interest, the lowest k value which is allowed for the first-eluting peak and the required minimum resolution all need to be estimated. If the number of components is not known, but gradient data are available, the total number can be estimated from the statistical theory of component overlap devised by Martin *et al.* [30].

2.2.1. Transfer to other solvents. A useful addition to the above procedure is the possibility of transferring to an alternative organic modifier after satisfactory retention conditions have been established for the methanol–water system. Empirical transfer rules [25] can be used to estimate the appropriate compositions of binary acetonitrile–water and THF–water mixtures. These so-called iso-eluotropic mixtures are expected to yield approximately the same retention times, in combination with different (hopefully improved) selectivity. The equations were obtained for an "average" solute, based on experimental data for a large number of aromatic compounds. Within the range $1 < k < 10$ the following equations can be applied:

$$\varphi_{ACN} = 0.32\varphi^2_{CH_3OH} + 0.57\varphi_{CH_3OH} \tag{30}$$

where ACN is acetonitrile, and

$$\varphi_{THF} = 0.66\varphi_{CH_3OH} \tag{31}$$

The use of eqns. 30 and 31 may lead to differences in the experimentally observed capacity factors of up to 50% between the methanol–water system and the acetonitrile–water system and of up to 100% between methanol–water and THF–water. If the entire group of solutes in the sample is either eluted too early or too late, additional isocratic measurements can be performed as described by Herman *et al.* [31] and by Haddad and Sekulic [32]. Patel and Jefferies [33] have explored the possibility of using the octanol–water partition coefficient of the mobile phase as a solvent strength parameter. In their studies, the predicted iso-eluotropic compositions were in good agreement with those predicted by the above transfer rules (eqns. 30 and 31).

In Fig. 2 a graphical comparison is given for the equations derived by Schoenmakers *et al.* [25], Herman *et al.* [31] and Patel and Jefferies [33]. In the lower range of organic modifier concentrations (up to 40 or 50% methanol) all the equations predict similar iso-eluotropic eluent compositions. At high concentrations of organic modifier the transfer rules given by Schoenmakers *et al.* [25] are seen to yield values between the other two.

2.3. Single gradient scan plus isocratic correction

Because eqn. 27 will only be approximately valid, the predicted isocratic elution conditions may differ from those observed experimentally in a subsequent experiment. Deviations of up to a factor of two have been recorded, although the errors are usually less. The combination of the gradient elution retention data and those obtained in the first isocratic experiment allow a more accurate characterization of the isocratic retention behaviour. If we again use RPLC as our example and assume eqn. 16 to be valid, we have for the capacity factor (k_c) at the predicted isocratic composition (φ_c)

$$\ln k_c = \ln k_0 - S\varphi_c \tag{32}$$

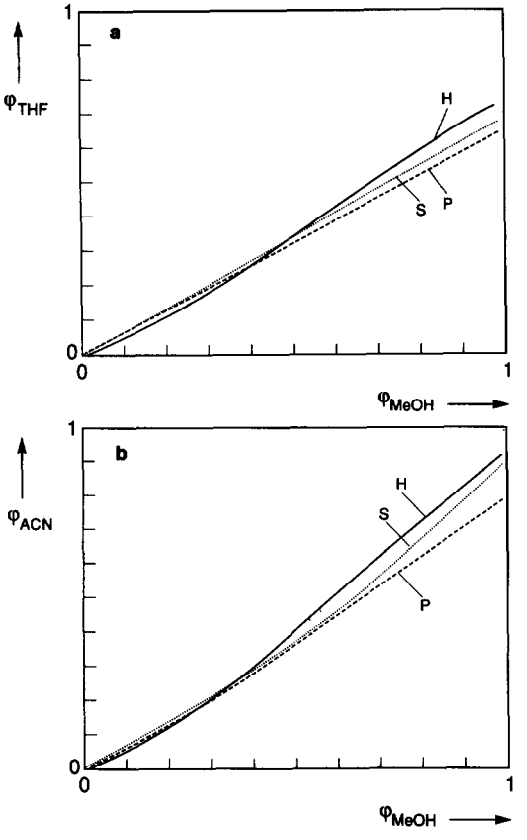

Fig. 2. Comparison of the transfer rules given by Schoenmakers *et al.* [25], Herman *et al.* [31] and Patel and Jefferies [33]. (a) Transferring from methanol to THF; (b) transferring from methanol to acetonitrile. MeOH = methanol.

and

$$\ln k_a = \ln k_0 - S\varphi_a = \ln k_c - S(\varphi_a - \varphi_c) \tag{33}$$

Substitution of eqn. 33 in eqn. 23 (injecting the sample at $t = \tau$) yields

$$t'_{R_i} = \frac{1}{Sb} \ln [1 + Sbt_0 k_c e^{-S(\varphi_a - \varphi_c)}] \tag{34}$$

Again, this equation cannot be made explicit in S, but it can easily be solved numerically. Once knowing S, the intercept $\ln k_0$ can readily be obtained by rearranging eqn. 32. A correction procedure based on this principle has been described by Herman *et al.* [31]. The practical correction method described by Haddad and Sekulic [32] for ionogenic solutes (see section 3.3) can also be used. Still more pragmatic approaches have been reported by Snyder *et al.* [34], based on the so-called linear solvent strength theory, and by De Smet *et al.* [35], who used expert systems.

3. IONIC SOLUTES

3.1. Selecting the optimum elution range

The optimization of the separation of sample mixtures containing ionic and/or ionogenic and neutral compounds represents a complex task owing to the large number of (often interrelated) mobile phase variables which affect solute retention and selectivity. In RPLC the separation of non-charged solutes involves almost exclusively the manipulation of the type and/or concentration of the organic modifier(s) in the aqueous eluents. The separation of samples containing ionic solutes usually needs the variation or correct selection of eluent pH, ionic strength, type and concentration of the ion-pairing reagent and the organic modifier(s).

An important feature of sample mixtures containing ionic solutes is that at a given (isocratic) concentration of the organic modifier the retention of all solutes, thus including the first- and the last-eluting ones, may be considerably influenced by the eluent pH or by the addition of ion-pairing reagents. When a sample contains only neutral compounds, a very large difference in the gradient retention times of the first- and the last-eluting peaks may clearly indicate that isocratic elution of the sample is not possible. On the other hand, when the first- and last-eluting compounds are different with respect to charge or type, varying the pH or adding a pairing ion may bring the first and last peaks closer together so as to make isocratic elution of the sample possible.

It must be pointed out that the charge and type of the components play an important role in the selection of the optimum capacity factor range and the corresponding organic modifier concentration of the isocratic eluent. Let us consider a four-component mixture of two neutral solutes and two strong bases, with a large difference in the retention times between these two groups of solutes. The hypothetical $\ln k$ *vs.* organic modifier concentration (φ) behaviour for the four components is shown in Fig. 3a. If the strong bases elute as the last two peaks in the chromatogram, then the two groups of peaks can be brought closer by decreasing the retention of the late-eluting peaks. This may be achieved by adding a similarly (positively) charged pairing ion (see Fig. 3b). The organic modifier concentration must be fixed at a level (φ_1) which results in reasonable retention times for the two hydrophilic neutral solutes. If the sample contains two hydrophilic strong bases and two late-eluting neutral components (see Fig. 3c), then the organic modifier concentration must be high enough (φ_2) to elute the last peaks within an acceptable analysis time. The retention of the early eluting strong bases can be increased by the addition of an oppositely (negatively) charged ion-pairing reagent.

In both instances, the cluster of the neutral solutes must be considered in selecting the optimum k range. The retention of the ionic compounds may be out of the optimum range in this initial chromatogram, but it can be increased or decreased by at least one order of magnitude by adding a pairing ion of the appropriate hydrophobicity and concentration [36]. Once the retention of all components falls within the desired range, systematic selectivity optimization can be carried out (*e.g.*, by varying the type of the organic modifier [37]).

Obviously, these two hypothetical samples represent different separation problems and require different initial isocratic concentrations of organic modifier for a successful optimization of the separation, even though the $\ln k$ *vs.* φ behaviour (and

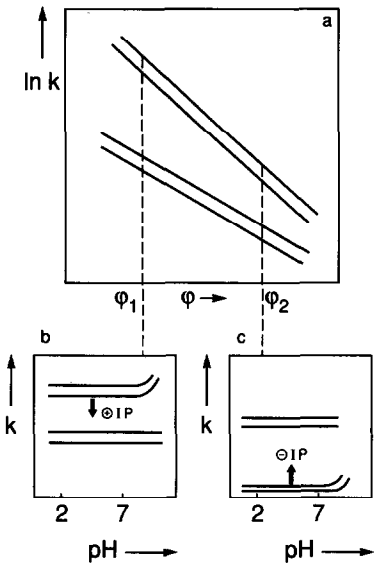

Fig. 3. (a) Schematic k *vs.* φ behaviour of a four-component sample mixture; (b) schematic k *vs.* pH behaviour of a sample mixture containing two hydrophobic strong bases (SB) and two hydrophilic non-charged (N) solutes at a constant organic modifier concentration (φ_1); (c) schematic k *vs.* pH behaviour of a sample mixture containing two hydrophobic non-charged (N) solutes and two hydrophilic strong bases (SB) at a constant organic modifier concentration (φ_2). Arrows indicate the direction of retention changes when an ion-pairing reagent (IP) is added to the eluent.

the gradient retention times) in the two cases may be identical. Therefore, the information on the charge and type of the solutes and the retention times of both the first- and the last-eluting solute peak(s) must always be carefully considered when deciding upon a suitable isocratic composition from gradient data.

When the sample contains components of other types (*e.g.*, weak acids or bases), the effect of the eluent pH must also be considered. For complex sample mixtures practical parameter selection rules [36–38] and expert systems [39–41] are valuable tools for guiding the analyst in the selection of suitable initial experimental conditions.

In order to make sound decisions, information is needed about the charge and type and the relative hydrophobicity (relative retention) of the components. When the nature of the sample components is not known *a priori*, the information can be determined from a number of specifically designed organic modifier gradients.

3.2. Classification according to charge and type

In the early 1980s, Berry and Shansky [42–44] introduced the technique of "pulse injection" of ion-pairing reagents in combination with linear solvent strength gradients at constant eluent pH. The basis of this technique is to deposit a concentrated plug of ion-pairing reagent on the top of the reversed-phase column before sample injection and the start of the gradient. The hydrophobic ion-pairing reagent adsorbs on the column and alters the retention of the ionic sample components through ionic attraction or repulsion. As a result, the retention time of solutes with a charge opposite

to the ion-pairing reagent increases relative to the non-charged solutes and that of similarly charged solutes decreases.

The pairing-ion pulse-injection technique has been used for the separation of several complex sample mixtures [45]. Although its potential for obtaining information about the charge and type of the sample components has been recognized [26], until recently the method has not been used as a systematic scouting procedure prior to selectivity optimization.

Low *et al.* [38] developed a strategy to establish the types and charges of solute ions from the retention shifts of the sample components that were observed in four successive 0–90% (v/v) methanol–buffer gradients run at pH 2.5 and 7.5. In two of the runs pairing-ion plugs, containing octanesulphonate (pH 2.5) or tetrabutylammonium (pH 7.5), respectively, were injected prior to sample injection. By tracking the shifts of the peaks in the different chromatograms, type assignments can be made by comparing the observed shifts with the expected (ideal) behaviour of charged solutes [38,46,47]. A typical example of solute-type determination by the above strategy is presented in Fig. 4 [47]. Four gradient chromatograms of a reaction mixture of pyrroloquinoline-quinone (PQQ) and cyclopropanol were recorded at different combinations of pH and pairing-ion pulse injection. It is important to note that only PQQ and its 5-(3-propanal) adduct (PQQ-M) were known in the sample mixture, and that no reliable information on the number and type of the other reaction products was available.

When the two gradients at pH 2.5 without (Fig. 4a) and with (Fig. 4b) octanesulphonate pulse injection are compared, no significant retention shifts are seen, indicating that all solutes are in a non-charged form at this eluent pH. In the gradient

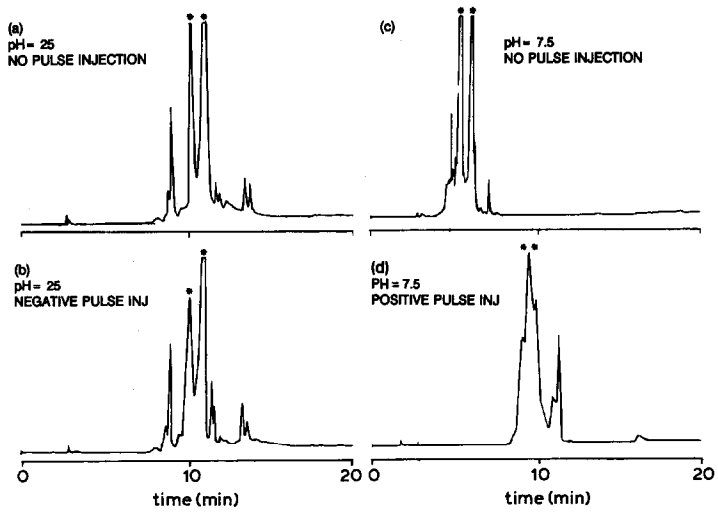

Fig. 4. Gradient elution chromatograms of a PQQ reaction mixture. Linear gradients of 0–90% methanol–triethylamine phosphate buffer in 15 min. (a) pH 2.5 without pulse injection; (b) pH 2 with pulse injection of negatively charged octanesulphonate (as sodium salt); (c) pH 7.5 without pulse injection; (d) pH 7.5 with pulse injection of positively charged tetrabutylammonium (as bromide). Column, 20 cm × 4.6 mm I.D., packed with 5-μm Hypersil ODS. Flow-rate, 1.0 ml/min (t_0 = 2.05 min); UV detection at 320 nm. Asterisks indicate positions of PQQ and PQQ-M. Reprinted from ref. 47 with permission.

chromatogram at pH 7.5 without pulse injection (Fig. 4c) the retention times of all peaks are shorter by about 50%. The last peak observed with the pH 7.5 gradient even appears before the first one in the pH 2.5 gradient. This can be explained only by a lower hydrophobic retention of the ionized (dissociated) form of weakly acidic groups. Therefore, after examining three gradients we already know that all solutes are weak acids. However, we do not know whether all contain the same number of charged groups, as one or two negative charges may cause equally early elution in the gradient at pH 7.5. The pulse injection of a positively charged pairing-ion at pH 7.5 (Fig. 4d) results in a collective shift of all peaks to higher retention, indicating that all solutes have equal negative charge(s). Based on the information obtained from these scouting experiments, the relevant optimization parameters were selected and a successful iterative optimization was performed (see ref. 47 for details).

The main advantage of this experimental strategy is that it allows a rapid re-equilibration of the chromatographic system and a flexible variation of experimental conditions with different pH and pairing-ion combinations. Berry [45] has also shown that with sufficient purity of the mobile phase components and pairing-ions, gradients can be run using "near-universal" detection, *i.e.*, UV absorption at wavelengths down to 210 nm. There are two experimental factors which have to be carefully adjusted in order to classify the solutes based on the four gradients. First, the behaviour of non-charged compounds, strong acids and strong bases must be approximately ideal, *i.e.*, retention must be largely independent of variations in the pH and the buffer composition. This can usually be achieved by a careful selection of the stationary phase and by using a triethylamine–phosphate buffer [38,48,49]. Second, retention shifts induced by the pairing-ion must occur for all ionic solutes, irrespective whether they elute early or late in the gradient run. However, the adsorbed pairing-ion is increasingly removed from the column in the later part of the organic modifier gradient. Therefore, its effect diminishes for the late-eluting solute ions. In order to avoid early elution of the pairing-ion plug, multi-component pairing-ion mixtures can be applied [50].

When the retention shifts of the individual sample components can be established from the different chromatograms, classification of the solutes is straightforward [38,46]. However, peak tracking by injecting standards is either impractical or even impossible in the case of unknown samples. Peak tracking based on UV spectra (using a diode-array detector) is hampered by the sometimes dramatic variations in the spectral properties of ionogenic compounds with eluent pH [38]. Therefore, an extended design of seven linear gradients (run at pH 2.5, 5 and 7.5, with and without pulses of positively and negatively charged pairing-ions) in combination with an "artificial intelligence"-type computer programme has been proposed for establishing solute types without peak tracking. It has been demonstrated that solute classification can be carried out by this strategy for totally unknown sample mixtures [50].

3.3. Isocratic conditions for ionic solutes

Once the sample components are classified (either on the basis of *a priori* information or by means of a gradient scouting procedure) the results of gradient scans can be used to define non-programmed mobile phase compositions, where the sample mixture can be eluted in the selected optimum range. This is a vital step in the

application of various selectivity optimization procedures, *e.g.*, those described by Goldberg *et al.* [48], Coenegracht *et al.* [49] and Billiet *et al.* [51].

In reversed-phase chromatography the translation of the results of the gradient scans into isocratic eluent compositions is often based on some general assumptions for the approximate value of the slope (S) of the relationship between retention ($\ln k$) and the volume fraction of organic modifier (φ; see section 2.2). However, the gradient scouting procedures used for non-charged solutes often fail to provide good estimates of isocratic eluent compositions for ionic solutes [46,47]. Limited sets of data [52,53] suggest that in water-rich eluents and with comparable retention the slope of the $\ln k$ *vs.* φ relationship is generally steeper for ionic solutes than it is for non-charged solutes. In Fig. 5 the $\ln k$ data of fully ionized strong acids, bases and non-charged solutes are plotted as a function of the concentration of methanol and acetonitrile in an aqueous phosphate buffer eluent (see ref. 53 for experimental details). The differences in the slopes for ionic (solid lines) and non-charged (dashed lines) solutes might at least in part be responsible for the failure of conventional methods to predict suitable isocratic conditions for the elution of sample mixtures containing ionic solutes. Thus, one should expect large deviations between the observed k values of ionic solutes and the predicted values in the first isocratic binary eluent. Rules of thumb given by Snyder *et*

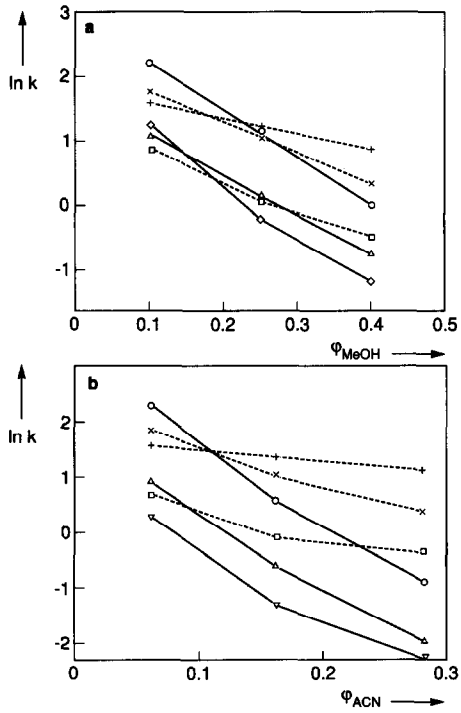

Fig. 5. Retention (k) of non-charged (dashed lines) and ionic (solid lines) solutes as a function of the concentration of (a) methanol and (b) acetonitrile in an aqueous 50 mM phosphate buffer eluent (pH 2.1) on a Hypersil ODS (5-μm) column. Solutes: + = methyl iodide; □ = 2-butanone; × = phenol; ○ = 2-naphthalenesulphonic acid; △ = phenylalanine; ◇ = morphine; ▽ = isoprenol. See ref. 53 for further details.

al. [34] or experimental procedures, such as that described by Haddad and Sekulic [32], can be applied to readjust the concentration of the organic modifier and to elute the sample mixture within the required retention limits.

The method suggested by Haddad and Sekulic [32] is based on a simple stepwise approximation of the capacity factor of the last-eluting peak using experimental isocratic retention data. The advantage of this procedure is that it can easily be extended [54] to estimate the retention of both the first- and the last-eluting sample components. A schematic illustration of this extended method is shown in Fig. 6. First, one should decide on the target capacity factors for the first (k_α^*) and the last (k_ω^*) eluting peaks. Maximum acceptable differences between the required and the actual solute retentions (*e.g.*, 20%; indicated by the horizontal dashed lines in Fig. 6) can also be defined. Next, the capacity factors of the first (k_{α_1}) and the last (k_{ω_1}) peaks obtained with the first isocratic eluent (φ_1) are compared with the target values. If the measured values fall outside the shaded area, an estimate is made of a new organic modifier concentration (φ_2) by assuming a linear ln k *vs.* φ relationship and a steep slope (*e.g.*, $S = 20$ in the reversed-phase mode). The sample mixture is chromatographed again at φ_2, and the capacity factors of the first (k_{α_2}) and the last (k_{ω_2}) peaks are determined. These are compared again with the target values (k_α^* and k_ω^*). If the deviations still exceed the predefined limits, the k values from the two isocratic measurements can be used to calculate new slope values and to estimate a new mobile phase composition (φ_3). This can be done so as to obtain the target value for the first- or for the last-eluting peak using either of the following equations:

$$\varphi_3^{<\alpha>} = \left(\frac{\varphi_1 - \varphi_2}{\ln k_{\alpha_1} - \ln k_{\alpha_2}}\right)(\ln k_\alpha^* - \ln k_{\alpha_1}) + \varphi_1 \tag{35}$$

or

$$\varphi_3^{<\omega>} = \left(\frac{\varphi_1 - \varphi_2}{\ln k_{\omega_1} - \ln k_{\omega_2}}\right)(\ln k_\omega^* - \ln k_{\omega_1}) + \varphi_1 \tag{36}$$

The next isocratic composition will be a compromise (often the average) between the values of $\varphi_3^{<\alpha>}$ and $\varphi_3^{<\omega>}$.

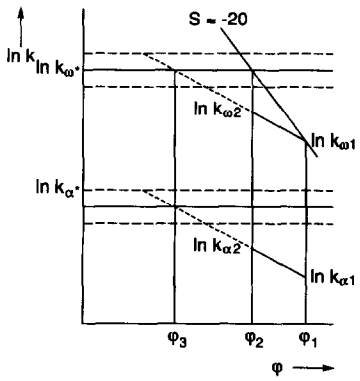

Fig. 6. Schematic representation of the stepwise approximation procedure of Haddad and Sekulic [32], extended to estimate the retention of both the first- and the last-eluting components of the sample.

4. CONCLUSIONS

Gradient-elution methods can elegantly be used for rapidly establishing the appropriate isocratic elution conditions for newly chromatographed samples. In comparison with the conventional approach of running a number of isocratic chromatograms on a trial-and-error basis, gradient-elution methods can be much more efficient and yield more consistent results.

Key factors are (i) a description of the retention behaviour of the solutes under isocratic conditions as a function of the programmed parameters and (ii) accurate knowledge of the actual gradient profile, *i.e.*, the variation of the programmed parameters with time. Reasonably simple calculation procedures are facilitated by the use of simple (*e.g.*, linear) programs and instrumentation that affects the actual profile as little as possible.

For non-ionic solutes two different gradient scans suffice in principle. In certain cases a single gradient scan may be adequate. A combination of one gradient scan and one isocratic verification experiment can often be used for the accurate prediction of optimum isocratic conditions.

For ionic solutes a larger number of scanning experiments is needed than for non-ionic solutes, but in comparison to the time needed for trial-and-error optimization the potential benefits of gradient-scanning methods are much greater. For characterizing the ionic solutes in unknown samples two things are needed. First, the solutes need to be classified according to their type (weak or strong; acids or bases) and charge. Next, the optimum isocratic conditions should be established. The concentration of organic modifier, the pH, and the type and concentration of ion-pair reagent are the most important parameters to be considered in the process. Using linear gradients at two different pH values in combination with "pulse injection" of ion-pairing reagents, four scanning experiments form the basis of an efficient classification procedure. Once the solutes have been classified, simple, stepwise procedures can be used to establish optimum isocratic conditions.

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