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PREDICTIVE CALCULATION METHODS FOR OPTIMIZATION OF GRADIENT ELUTION USING BINARY AND TERNARY SOLVENT GRADIENTS

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SUMMARY

Predictive optimization methods in gradient elution liquid chromatography are reviewed. The optimization approach is based on calculations of elution volumes and band widths in various modes of gradient elution liquid chromatography. The calculation makes use of the parameters of retention–mobile phase composition equations determined in a few preliminary isocratic experiments.

In liquid chromatography with binary solvent gradients, either a direct calculation method may be used to optimise the gradient profile in order to achieve a desired resolution of a "critical pair" of sample solutes, or "maps" of the dependence of resolution of the individual pairs of compounds on gradient steepness and initial mobile phase composition can be constructed for an assumed constant gradient volume (time).

Calculation methods are also applicable to ternary mobile phase gradients in reversed-phase systems. Here, ternary "solvent strength", "selectivity" or "combined selectivity–solvent strength" gradients are chosen, depending on the separation problem to be solved. In these systems, construction of resolution "maps" is the method of choice for predictive optimization.

The precision of predicted gradient elution data depends on the instrumentation used and on its ability to reproduce accurately the gradient profile programme and on possible deviations of the experimental retention–mobile phase composition plots from the theoretically expected forms of these dependences. The effect of the choice of gradient shape (curvature) and volume on the optimized separation is also addressed.

INTRODUCTION

Gradient elution (or solvent-programmed) liquid chromatography is a preferred technique used to improve the separation of sample mixtures with a wide retention range[1]. Isocratic separations of such mixtures usually lead either to an incomplete resolution of the early eluted solutes or to excessive elution times of sample compounds with a great affinity to the stationary phase. Even the separation of some samples with a limited retention range may be significantly improved using gradients with judiciously adjusted profiles, much like the selectivity of isocratic separation can often

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be improved by optimization of the concentration ratio of the individual mobile phase components. With increasing complexity of samples to be analysed, high-performance liquid chromatographic (HPLC) gradient elution is being applied more and more frequently and is becoming an almost indispensable tool in HPLC method development. This has a direct impact on the need for adequate methods for the optimization of the profile of solvent gradients.

A variety of modern instruments for HPLC are equipped with a standard or optional possibility for working with binary, ternary, quaternary or even more complex solvent gradients. Each mobile phase component may change its concentration with time according to a programme in the form of a continuous straight or curved line or of a set of subsequent steps (segments) in each of which the mobile phase may either be kept constant or changed continuously in a linear or non-linear manner, depending on the type of liquid chromatograph used.

Most practical separation problems can be solved using binary gradients, although ternary gradients may be useful for better control of the separation selectivity and even quaternary gradients have been suggested for this purpose[2]. In addition to the imperfect empirical "trial-and-error" approach to the selection of the gradient profile, systematic optimization methods have been developed in recent years.

The simplex optimization procedure has become popular, because it does not require an understanding of the principles of the separation mechanism and essentially the same strategy is applicable for the optimization of the initial and final compositons of the mobile phase and of the slope and shape of a solvent gradient, as for the optimization of a three-component mobile phase composition in isocratic chromatography[3].

The "Sentinel" method, originally introduced for the optimization of selectivity in isocratic chromatography with quaternary mobile phases, was later extended to the optimization of quaternary gradients[2]. This method assumes "iso-eluotropic" initial and final mobile phase compositions and a linear change in elution strength between the two compositions during the gradient run. The resolution of the individual pairs of sample compounds is "mapped" as a function of the changing ratio of the solvents at the start and at the end of the gradient in seven independent experiments and optimum initial and final mobile phase compositions are determined after quadratic interpolation between the data points from the individual experiments[2].

Predictive optimization of gradient elution chromatography is based on calculations of the solute elution volumes, bandwidths and resolution expected at various gradient profiles from the data acquired in a few preliminary isocratic or gradient experiments. Theoretical equations derived on the basis of retention models in various modes of HPLC are used for this purpose.

The widespread availability of personal computers in recent years has led to a major breakthrough in the optimization of chromatographic separations. Not only the statistical simplex optimization but also the predictive optimization of either isocratic or gradient elution chromatography have significantly benefited from the use of computers, as a great number of direct optimization calculations or predictive simulations can be performed in a short time, the results can be automatically sorted and compared and the best solution selected. This spares much time and expense connected with the experimental trial-and-error optimization approach.

Simple calculations can be used for so-called "linear solvent strength" gradients

developed by Snyder[4], where the profile of a gradient is controlled by a linear increase in the logarithms of solute capacity factors (instantaneous) in the mobile phase at the column inlet. In the real world, linear concentration gradients of an organic solvent in water in reversed-phase systems are fairly close to this model. For these gradients the optimum gradient slope that is most likely to give the best separation of an unknown sample mixture can be calculated[5,6]. The initial composition of the mobile phase and the gradient time can be optimized using a trial-and-error approach[5,6]. Dry-Lab G is a much more sophisticated predictive approach based on Snyder's theory.[7]

We have earlier proposed several predictive optimization procedures for binary, ternary and stepwise gradient elution chromatography applicable in reversed-phase, normal-phase and ion-exchange systems[8–13]. In this paper, these methods are reviewed and compared and their limitations are discussed.

PREDICTION OF RETENTION CHARACTERISTICS IN GRADIENT ELUTION LIQUID CHROMATOGRAPHY BY CALCULATION

Binary solvent gradients

Calculations of elution volumes in gradient elution chromatography are based on the solution of the fundamental equation describing the distribution of a solute between the stationary and mobile phases characterized by the capacity factor, k', of a sample solute. k' does not change during the migration of the solute band by a distance corresponding to a differential fraction of the column dead volume, dV_m, caused by a differential increase in the volume of the mobile phase that had passed through the column, dV:

$$dV = k' dV_m \tag{1}$$

This differential equation may be solved by integration in the limits from 0 to V_m and from 0 to V'_g, where V'_g is the net elution volume of a sample solute in gradient elution chromatography. Eqn. 1 or its slightly modified forms have been introduced and solved by Drake[14] and other workers[15–17]. The dependence of the solute capacity factor on the volume of the eluate, V, should be known and formulated in a relatively simple mathematical form, otherwise the explicit solution of eqn. 1 for elution volume, V'_g, is not feasible. For a "linear solvent strength" gradient this dependence is described by a simple equation 2[4,5,16]:

$$\log k' = \log k'_a - \beta V \tag{2}$$

where k'_a is k' at the beginning of the gradient elution and β is the slope of the gradient. Although the concept of "linear solvent strength gradients" is attractive in its simplicity, it is an idealization of reality as different compounds have different slopes, β, and this approach (*i.e.*, preselection of an average "best" value of β for every sample) can be used only for approximate predictions.

We have adopted another approach, applicable also to other types of gradients[17]. Here the depedence of k' on V is separated into two partial functions. The first describes the dependence of k' on the concentration of a more efficient eluting component in the mobile phase, φ, which is controlled by the nature of the sample

solute and by the chromatographic system used and the other, so-called "gradient function", is the dependence of φ on V which controls the gradient profile. It is advantageous to select the form of the "gradient function" in such a way that it is characterized by three parameters, namely the initial concentration of the stronger eluent in the mobile phase at the start of the gradient, A, the slope of the gradient, B, and its shape (curvature parameter), κ. It should be noted that generally only gradients with increasing concentration of the stronger eluent, *i.e.*, with increasing elution strength, are useful in practice, with the exception of the "selectivity gradients" where the elution strength is kept approximately constant.

The relationship between the solute capacity factor, k', and the composition of a binary or multi-component mobile phase is determined by the mode of liquid chromatography and by the retention mechanism controlling the separation. The mathematical form of the equation describing this relationship may be complex, but only simple forms of these equations are suitable for the calculation of retention data and predictive optimization of gradient elution chromatography[10]. A more detailed discussion of this topic would be beyond the scope of this paper and can be found in refs. 1, 10 and 18–24. The possibilities for explicit and implicit solutions of eqn. 1 for various combinations of log k' *versus* φ relationships and "gradient functions" describing the gradient profile are discussed in detail elsewhere[10].

In reversed-phase chromatographic systems, the log k' *versus* φ relationship is more accurately described by a quadratic equation[10,17,25], but a simple linear equation (eqn. 3) is often satisfactory for describing the retention behaviour of numerous solutes, mainly in chromatographic systems with methanol–water mobile phases[1,5,10,17,25,26]:

$$\log k' = a - m\varphi \tag{3}$$

where φ is the volume concentration of the organic solvent in the aqueous–organic mobile phase and a and m are experimental constants depending on the structure of the solute, on the nature of the organic solvent used and on temperature.

A linear binary gradient of increasing concentration of the organic solvent in water is described by

$$\varphi = A + BV \tag{4}$$

where V is the volume of the eluate passed through the column from the start of the gradient, A is the initial concentration of the organic solvent in the mobile phase and B is the slope of the gradient in concentration units per 1 ml of the eluate. In reversed-phase systems with linear solvent gradients, the logarithms of solute capacity factors decrease linearly with increasing volume of the eluate, provided that eqn. 3 is suitable for the description of solute retention:

$$\log k' = a - mA - mBV \tag{5}$$

In this instance the integration of eqn. 1 yields the following expression for the elution volume in gradient elution chromatography, V_g[1,10,17,26]:

$$V_g = \frac{1}{mB} \cdot \log \left[2.31 mBV_m \cdot 10^{(a - mA)} + 1 \right] + V_m \tag{6}$$

This equation applies provided that there is no significant gradient dwell time in the instrument, *i.e.*, the gradient delay between the gradient mixer and the top of the column is negligible.

Rigorous calculations of solute band widths in gradient elution chromatography, w_g, are possible using fairly complex expressions, which are not very practical. With some simplification, the width of a solute band eluted under gradient conditions can be considered to be approximately equal to the band width in isocratic chromatography with a mobile phase of the same composition as the instantaneous composition in gradient elution chromatography at the time of elution of the maximum of the solute band[17]. For linear binary gradients in reversed-phase systems, this treatment leads to eqn. 7, provided that eqn. 3 applies:

$$w_g = \frac{4V_m}{\sqrt{N}} \left\{ 1 + \left[2.31 m B V_m + 10^{(mA - a)} \right]^{-1} \right\} \tag{7}$$

where N is the column plate number under isocratic conditions, which is assumed not to depend very significanly on the mobile phase composition, φ, *i.e.*, on the solute capacity factor, k'. It should be remembered that the well known equation for plate number:

$$N = 16 \left(\frac{V_R}{w} \right)^2 \tag{8}$$

applies only under isocratic conditions, where the solute capacity factor, k', is constant, and cannot be used for gradient elution chromatography.

The simplified eqn. 7 neglects an additional compression of the solute band resulting from the fact that the front of the band moves in the mobile phase with a lower elution strength than the end of the band and from an anomalous broadening of bands when steep gradients are used[1,4,27]. However, the last two effects tend to compensate each the other and can be taken into account, if necessary, by multiplying the band widths calculated using eqn. 7 by an empirical factor of 1.1 as a "security measure"[1,7].

In normal-phase chromatography on polar adsorbents, the log k' *versus* φ relationships can be most simply described by

$$\log k' = \log a - m \log \varphi \tag{9}$$

where φ is the concentration of the more polar organic solvent in a binary organic mobile phase and the meaning of the constants a and m is as in eqn. 3. Eqn. 9 can often also be used in the ion-exchange chromatography of completely ionized solutes, with φ being used for the molar concentration of a competing ion (salt) in the mobile phase[10,25]. For solvent gradients controlled by the general gradient function[17]

$$\varphi = (A^{\frac{1}{\kappa}} + BV)^{\kappa} \tag{10}$$

eqn. 1 can be integrated to yield the following expression for the elution volume of

a sample solute in normal-phase gradient elution chromatography, provided eqn. 9 is valid[17]:

$$V_g = \frac{1}{B}\left[(\kappa m + 1)BaV_m + A^{\frac{\kappa m+1}{\kappa}}\right]^{\frac{1}{\kappa m+1}} - \frac{A^{\frac{1}{\kappa}}}{B} + V_m \qquad (11)$$

Eqn. 10 is used to describe linear or curved (convex and concave) gradient profiles. The meaning of the parameters A and B is the same as in eqn. 4; κ is the curvature parameter defining the shape of the gradient profile[17].

Using the same approach and simplifying assumptions as in reversed-phase gradient elution chromatography, the expression for the solute band width, w_g, was derived in the form[17]

$$w_g = \frac{4V_m}{\sqrt{N}}\left\{1 + a\left[A^{\frac{\kappa m+1}{\kappa}} + (\kappa m + 1)BaV_m\right]^{-\frac{\kappa m}{\kappa m+1}}\right\} \qquad (12)$$

More complex solvent gradients

Ternary (and quaternary) mobile phases composed of water and two or three organic solvents have been recommended for the control of separation selectivity in reversed-phase chromatography by adjusting the concentration ratio of the organic solvents[28–30]. As in reversed-phase chromatography with binary mobile phases, the solute capacity factors in multicomponent mobile phases can be calculated as a function of the volume concentrations, φ_i, of the individual organic solvents[31,32]. After neglecting second-order terms, a simplified equation for k' in ternary mobile phases composed of water and two organic solvents X and Y (such as methanol, acetonitrile or tetrahydrofuran) in concentrations φ_x, φ_y, can be written as[11,13]:

$$\log k' = \frac{a_x\varphi_x + a_y\varphi_y}{\varphi_x + \varphi_y} - m_x\varphi_x - m_y\varphi_y \qquad (13)$$

where a_x, m_x, a_y and m_y, are the constants a and m in eqn. 3, measured in binary mobile phases containing water and only one organic solvent, X or Y, respectively. Hence eqn. 13 can be used for approximate predictive calculations of retention in reversed-phase chromatography with ternary mobile phases from the data measured in binary mobile phases[33].

For gradient elution with linear ternary gradients of both X and Y according to the partial gradient functions

$$\varphi_x = A_x + B_xV; \qquad \varphi_y = A_y + B_yV \qquad (14)$$

the same derivation approach was adopted as for binary gradients to yield the following expression for the elution volume[11]:

$$V_g = \frac{1}{m_xB_x + m_yB_y} \cdot \log\left[2.31V_m(m_xB_x + m_yB_y) \cdot 10^{(a_G - m_xA_x - m_yA_y)} + 1\right] + V_m \qquad (15)$$

where a_G represents the mean value of the parameters a_x and a_y during the gradient elution:

$$a_G = \frac{\left(A_x + B_x \cdot \frac{V_g}{2}\right) a_x + \left(A_y + B_y \cdot \frac{V_g}{2}\right) a_y}{A_x + A_y + (B_x + B_y) \frac{V_g}{2}} \quad (16)$$

V_g can be calculated from eqns. 15 and 16 with the parameters a_x, m_x, a_y and m_y determined experimentally in isocratic binary mobile phases. An iterative method should be used for this purpose, with the aid of a simple computer program, the elution volumes V_g are usually calculated with a precision of 1% relative using only 4–7 iterations[11].

Band widths are calculated from

$$w_g = \frac{4V_m}{\sqrt{N}} \cdot (1 + k'_f) \quad (17)$$

after the introduction of eqn. 13 for φ_x and φ_y corresponding to the volume of the eluate at the time of elution of the band maximum, *i.e.*, $V = V_g$; k'_f is the k' value of a solute at the time of elution.

OPTIMIZATION OF THE PROFILE OF A BINARY SOLVENT GRADIENT

General aspects of predictive optimization

When attempting to apply the predictive optimization of gradient elution chromatography, we should distinguish two different cases. In the first, we do not have much information about the chromatographic behaviour and often even about the number of sample compounds. In such a situation, the theory of linear solvent strength gradients estimates an optimum gradient steepness parameter $\beta(\beta V_m \approx 0.2)$, which is likely to yield maximum resolution per unit time on a column of a given length[5,6]. For a conventional analytical column (300 × 4 mm I.D., $V_m \approx 3$ cm^3) in reversed-phase chromatography of low-molecular-weight compounds (average $m = 3$ in eqn. 3), this corresponds to an increase of approximately 2% of the organic solvent in the mobile phase per 1 ml of the eluate.

A similar approach can be used in the reversed-phase chromatography of a mixture of compounds with a regular structural increment, such as in homologous or oligomeric series. Here, the separation selectivity between the neighbouring members of a given series is usually approximately constant under isocratic conditions[34]. Provided there are approximately constant band widths under gradient conditions and a low initial concentration of the organic solvent in the mobile phase, the gradient slope B (eqn. 4) necessary to obtain a desired resolution, R_{gd}, on a column with N theoretical (isocratic) plates and dead volume V_m can be calculated using

$$B = \frac{\sqrt{N} \cdot \log \alpha - 1.73 R_{gd}}{4mV_m R_{gd}} \quad (18)$$

where α is the average selectivity, *i.e.*, relative retention for neighbouring members of a given series, and m is the average slope of the log k' *versus* φ dependence (eqn. 3) in this series. For example, with a conventional analytical column (300 × 4 mm I.D., $V_m = 3\,cm^3$, $N = 3000$), eqn. 18 yields a gradient slope B of about 0.015–0.03 (1.5–3% of the organic solvent per 1 ml of the eluate) to achieve a resolution R_{gd} of 1.5–2 for various homologous series and solvents. For further details, see ref. 1, p. 172.

The above methods for the optimization of gradient steepness do not take into account the individual chromatographic behaviour of the solutes to be separated and also neglect the effect of the initial mobile phase composition and gradient shape on separation. More precise, "tailor-made" predictive optimization of gradient profiles can be accomplished provided that the dependence of k' of the sample solutes on φ can be described by a simple equation (*e.g.*, eqn. 3 or 9) and if the parameters of this relationship (a and m) for the individual sample solutes are known. These parameters have to be determined in preliminary isocratic experiments at two to four different mobile phase compositions; gradient "scouting" experiments may also be used for this purpose. The preliminary experiments are most convenient with pure standard compounds, but are also feasible with the sample mixture if the number of compounds is known. Because of possible changes in selectivity with changing mobile phase composition, which may lead to peak overlapping and even to a changed elution order, we should be able to identify the individual compounds. This identification can be based on spectral data, such as on the UV spectra obtained from a diode-array detector, or on the ratio of detector signals at different conditions of detection, *i.e.*, on the signals from two different detectors connected in series or on the signals at two different wavelength settings of a UV detector.

If we are working with a sample mixture of a constant composition, another useful aid for identification may be utilized. At a constant flow-rate, the peak areas are approximately independent of the mobile phase composition if a constant mass of sample is injected. Hence the ratios of peak areas in a given sample mixture should not depend very significantly on the mobile phase composition and may be utilized for partial solute identification in the preliminary "scouting" experiments. Also, if pure standards are available, it is not necessary to perform the "scouting" isocratic experiments with each of the standards separately, but one or a few artificial standard mixtures may be conveniently used for this purpose in connection with the above identification methods.

Simple mixtures of compounds with a relatively narrow retention range can usually be separated under isocratic conditions. A binary solvent gradient usually improves significantly the quality of separation and the analysis time for sample mixtures with a wide retention range, provided that the separation selectivity in the binary mobile phase is adequate.

Ideally, the peaks of all sample solutes should be regularly spaced with a desired resolution in the optimized chromatogram in the shortest time of separation possible. This can almost never be achieved in practice; optimized stepwise or segmented gradient elution chromatography may sometimes approximate this objective[9].

In continuous gradient elution chromatography, where the gradient profile is described by a single continuous curve, the slope B of the gradient and the initial mobile phase composition usually affect the separation more significantly than the shape (curvature) of the gradient[10,26]. A number of gradient liquid chromatographs

can use only linear gradient functions or segments. It seems more useful to start the computer-assisted optimization with a linear gradient than to optimize simultaneously the slope, shape and initial composition of the mobile phase at the start of the gradient. If the optimization results are unsatisfactory, the gradient curvature parameter can be re-adjusted and the optimization calculations repeated. A full simultaneous optimization of a binary solvent gradient is feasible using a statistical approach, but this necessitates a large number of experiments. On the other hand, both the elution time and separation selectivity depend significantly not only on the gradient slope, but also on the initial composition of the mobile phase at the start of the gradient, and consequently these two parameters should be optimized.

One optimization strategy for binary gradients proposed by Snyder[4] consists in optimizing the gradient slope in such a way that the average value of the sample solute capacity factor at the column midpoint is *ca.* 5. This should yield a compromise between good resolution and narrow bands for good detection with a reasonable time of analysis. Then, adequate peak capacity (at least 3–5 times greater than the number of components in the sample) is adjusted by controlling the gradient time, flow-rate, column length and gradient range —in other words, the gradient slope, B, which is a function of all these parameters[35]. Trial-and-error fine tuning of the above parameters (and hence of the gradient slope, B) can be used to influence the band spacing in the chromatogram so as to improve the overall resolution[36–38]. Finally,the initial concentration of the efficient eluting component in the mobile phase, A, can be optimized using a trial-and-error aproach[5,6].

This optimization strategy has been refined in the so-called Dry-Lab G system, which consists in predictive computer calculations of the retention characteristics for all sample solutes from the data acquired in two preliminary gradient experiments and predictive computation of simulated chromatograms for various gradient slopes and times. This allows the resolution of the poorest resolved band pair in the sample to be plotted *versus* the gradient time and the gradient slope and time that offer a satisfactory separation of the sample mixture to be selected (if this can be achieved). In the next step, the initial mobile phase composition and, if necessary, other experimental conditions can be optimized using trial-and-error predictive simulating computations to fine tune the separation[7]. In agreement with previous considerations[9], segmented gradients have been proposed as the most efficient means of achieving regular spacing of sample bands in the chromatogram[39,40].

The methods for the predictive optimization of binary gradients developed by ourselves[8,12] differ from the above optimization strategy principally in that both the slope of the gradient, B, and the initial mobile phase composition are optimized simultaneously, based on predictive calculations of the retention data with aid of a computer. For this purpose,the parameters of the k' *versus* φ equations measured under isocratic conditions in a few preliminary experiments are utilized. In these optimization methods the gradient range and time are determined by the initial concentration of the efficient eluent at the start of the gradient, gradient slope and the elution volume of the most strongly retained sample solute, after the elution of which the gradient is stopped.

In our predictive optimization methods, it is asumed that the column plate number does not depend very significantly on the type of the solute and on the mobile phase composition, *i.e.*, on the solute capacity factor. This is reasonably approximated

with most well designed chromatographic systems in contemporary HPLC. Further, under gradient conditions all sample solutes tend to be eluted with approximately equal instantaneous capacity factors at the time of elution of the band maximum, which diminishes significantly the practical importance of this effect[1,4].

Finally, it is assumed that the gradient device is able to reproduce accurately the pre-set gradient profile and that the gradient is not significantly changed by possible preferential sorption of some of the mobile phase components on the column packing material. The last effect is usually negligible in reversed-phase and ion-exchange chromatography, but it may become significant in normal-phase chromatography on polar adsorbents. These points are discussed in more detail in the last part of this paper.

We have developed two different strategies for computer-assisted predictive simultaneous optimization of the slope of a binary gradient and of the initial concentration of the stronger eluting component in the mobile phase at the start of the gradient.

Gradient optimization for a "critical" pair of solutes

In this optimization method[8], the parameters A and B of a gradient function with a pre-set shape (curvature) are optimized simultaneously so as to achieve the desired resolution for a "critical" pair of sample solutes j and k with adjacent bands that are most difficult to separate. At the same time, the elution volume V_{gi} of another adequately selected (usually the last eluted) compound i should be kept to a minimum, which should guarantee as short a separation time as possible.

From the mathematical point of view, the optimization method consists in the calculation of the minimum of the function $V_{gi} = f(A,B)$ at a given value of the curvature parameter, κ, for which the initial concentration A and the gradient slope B are interrelated by the condition that a desired value of the resolution R_{gd} should be obtained for the solutes j and k. Rather than the usual Lagrange method, the application of which for the solution of the problem would be connected with difficulties, a modified method of solution was suggested. The calculation is performed using a computer or a programmable calculator according to the scheme shown in detail in Appendix 1[8].

The optimization method assumes that there is only one minimum of V_{gi} in the interval of the A values. It can be applied universally with different $k' = f(\varphi)$ functions and gradient functions with a pre-set curvature parameter, κ, using different algorithms for computation. The gradient slope, B, cannot be calculated directly and iterative calculations using a computer are necessary. Hence a first estimate of the gradient slope, B_1, should be introduced among the input constants at the start of the calculation.

Another important point in this optimization approach is the selection of compounds i, j and k. The "critical" pair of compounds can be determined as that pair of sample solutes with adjacent bands yielding the lowest value of A_{max} calculated as the isocratic concentration φ necessary to provide just the resolution desired. Because the selectivity of separation changes to a certain extent with changing gradient parameters A and B (as it changes with changing mobile phase composition under isocratic conditions), it may occur that the originally selected "critical" pair of solutes is well resolved under the gradient conditions predicted by optimization calculation,

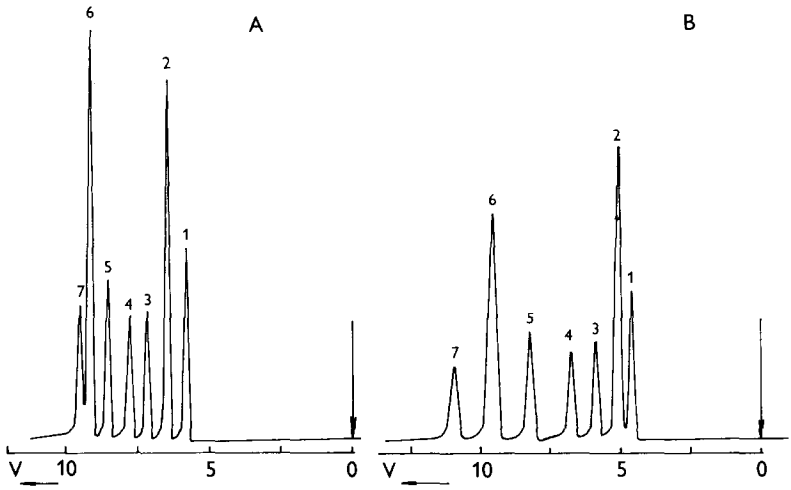

Fig. 1. Optimized reversed-phase separation of a mixture of seven barbiturates using a linear gradient of methanol in water. (A) $\varphi = 0.368 + 0.061V$; (B) $\varphi = 0.523 + 0.0082V$ (φ = concentration of methanol, vol-% · 10^{-2}; V = volume of the eluate, ml). Column: LiChrosorb C_{18}–Si 100, 10 μm (300 × 4.0 mm I.D.); flow-rate, 1 ml/min; detection, UV (254 nm). Solutes: 1 = barbital; 2 = heptobarbital; 3 = allobarbital; 4 = aprobarbital; 5 = butobarbital; 6 = hexobarbital; 7 = amobarbital.

but the resolution of another pair of sample compounds is impaired. Further, the selection of the compound *i* the elution volume of which should be minimized may affect the values of the optimized gradient slope, *B*, and the mobile phase composition at the start of the gradient, A, predicted by calculation. Consequently, the spacing of the peaks of sample solutes in the chromatogram depends to a certain extent on the choice of the compound *i*, which is usually the most strongly retained solute of the sample mixture[8]. This is illustrated by Fig. 1, showing the optimized gradient profile and resulting reversed-phase separation of a mixture of seven barbiturates on a LiChrosorb C_{18} column. On the basis of predictive calculations under isocratic conditions, the two least retained compounds, barbital and heptobarbital, were selected as the "critical" pair and the most strongly retained solute, amobarbital, as the compound *i* the elution volume of which should be kept to a minimum. A linear gradient of increasing concentration of methanol in water was optimized using eqns. 6 and 7 and the definition equation for resolution to yield the initial concentration of 37% methanol and the slope corresponding to the increase of 6.1% methanol per 1 ml of eluate. The separation was accomplished in 10 min, but now the resolution of the two last eluted compounds, hexobarbital and amobarbital, was not completely satisfactory (Fig. 1A). Therefore, the optimization calculations were repeated with the last two compounds as the "critical" pair and the least retained barbital as the compound *i*. The optimized gradient starting at 52% methanol with an increase of 0.82% methanol per 1 ml of eluate yielded satisfactory separation of all sample solutes in about 12 min (Fig. 1B). The agreement between the experimental and predicted elution volumes was better than 7% relative and also the resolution predicted corresponded well to the experimental evaluation of the chromatogram[8].

It is therefore recommended to perform the predictive optimization calculations with aid of a computer for all the potential "critical" solute pairs and at least for both the least and the most strongly retained sample solute selected as the compound i, the elution volume of which should be minimized; preferably all the sample solutes should be tested as the compound i. Then the results in the form of either a table of resolution between the adjacent solute bands or as the whole simulated chromatograms are compared to select the best predicted separation. The comparison can be performed automatically with the aid of a computer.

The optimization of complex sample mixtures should be better performed using the computer-assisted "resolution mapping" approach (see below).

Another important input constant for the computation is the desired resolution, R_{gd}, which is usually set equal to 1, 1.5 or 2, according to the objectives of the separation. It is sometimes impossible to achieve this resolution for one or more pairs of compounds under isocratic conditions on a given column. In this instance, the desired resolution also cannot be achieved in gradient elution chromatography and the calculation yields a negative value of A_{max}. In other separation problems the "critical" pair of compounds can be resolved only in too long a separation time. Here either a more efficient chromatographic column should be used, or another solvent, column or whole chromatographic system should be tested.

To elucidate the influence of the shape (curvature) of the gradient on the optimization procedure, the optimization calculations for the same practical example of the barbiturate mixture were repeated for a logarithmic (convex) gradient function:

$$\varphi = \log(A + BV) \tag{19}$$

which makes it possible to use eqns. 11 and 12 for the calculation of elution volumes and band widths in reversed-phase systems. The optimized profile of this logarithmic gradient was in surprisingly good agreement (better than 0.5% relative) with the optimized linear gradient used in the separation shown in Fig. 1B. This suggests that the preselected shape of the gradient is not very critical for the results of optimization, at least with relatively simple sample mixtures[8].

Optimization using "resolution mapping" at a constant volume (time) of the gradient

In this predictive optimization method, the gradient time, t_G, is pre-selected. The duration of the gradient determines the gradient volume, V_G, at a constant flow-rate of the mobile phase, F_m, $V_G = t_G F_m$. The gradient volume is the volume of the eluate from the start to the end of the gradient elution, which relates the gradient slope, B, to the concentration change from the initial, A, to the final, φ_G, concentration of the more efficient eluting component in the mobile phase. For a linear gradient

$$B = \frac{\varphi_G - A}{V_G} \tag{20}$$

The selection of V_G may appear arbitrary at first glance but, as will be shown in further discussion, it does not have a significant influence on either the quality or time of separation, provided that the gradient volume is sufficient to allow the elution of the most strongly retained sample solute. It should be noted that the gradient elution can

be stopped immediately after the elution of the most strongly retained compound, *i.e.*, at a volume of eluate lower than the preselected V_G. Hence for the purpose of the present optimization method V_G should be considered rather as a "working parameter" only, which need not necessarily correspond to the real volume of the eventually optimized gradient.

If constant values of V_G and φ_G are pre-selected, according to eqn. 20 each value of A determines simultaneously the gradient slope, B. After introduction of the appropriate expressions for elution volumes, V_G, and band widths, w_g, into the definition equation for resolution, the dependence of resolution on the initial mobile phase composition at a constant gradient volume can be predicted using computer-assisted calculations for each pair of solutes in the sample mixture[12]. This dependence can be presented either in tabular or in graphical form, which allows one to select the optimum initial concentration, A, of the more efficient eluting component and the corresponding gradient slope, B, at which the lowest value of resolution in the chromatogram is maximized. Instead of resolution, the differences in elution volumes of the compounds with adjacent peaks may be employed. These are more suitable than the ratios of net elution volumes (relative retention), which do not give a correct measure of separation selectivity under gradient conditions[1]. If the maximized minimum difference in elution volumes in the chromatogram is set equal to the band width of the later eluted solute from the pair concerned, the appropriate equation for band widths under gradient conditions, such as eqn. 7 or 12, may be used for the calculation of the plate number (isocratic) necessary to achieve the baseline separation of this solute pair under the optimized gradient conditions.

An important point in this optimization method is the selection of the gradient volume, V_G, and of the final concentration of the more efficient eluting component in the mobile phase at the end of the gradient, φ_G. Generally, both the maximum resolution of a given pair of sample solutes and the elution volumes corresponding to the optimized gradient conditions increase with increasing V_G. It has been verified that at a given value of desired resolution, R_{gd}, the elution volume of the most retained compound does not depend significantly on the pre-set value of V_G[13]. This means that the choice of V_G is not very critical for the results of optimization, provided a sufficiently large V_G is selected to allow for an adequate resolution of all sample solutes. The final concentration φ_G should be fitted to V_G so as to accomplish the elution of the last eluted sample compound in $V_g < V_G$. In this instance, the gradient need not necessarily be finished at the original V_G, but immediately after the elution of the last compound. In this way, the actual values of V_G and φ_G are diminished.

If the preset value of V_G is too low, the resolution desired cannot be achieved and the optimization procedure should be repeated. V_G should be selected with respect to the number of sample solutes to be separated (for example, 10–15 ml for 4–5 solutes, 30–40 ml for 8–12 solutes, etc., with a conventional analytical column).

This optimization approach can be illustrated by a reversed-phase chromatographic separation of a mixture containing 2,6- and 1,2-diaminoanthraquinones, 1- and 2-aminoanthraquinones, and anthraquinone on a 300 × 4.2 mm I.D. Silasorb C_{18} column using a linear gradient of 1,4-dioxane in water[13]. The separation was optimized so as to achieve a maximized minimum resolution in the sample mixture in 10 and in 15 ml of eluate with φ_G = 100% dioxane. The two resulting optimized chromatograms yielded similar resolution of the sample solutes in 9 and 10 ml of the eluate, respectively, as is shown in Fig. 2.

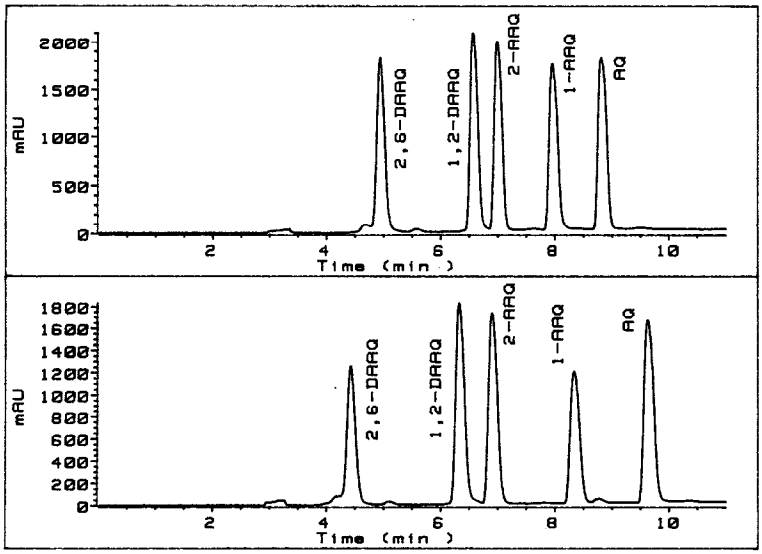

Fig. 2. Optimized reversed-phase separation of aminoanthraquinones using a linear binary gradient of 1,4-dioxane in water. Instrument: Hewlett-Packard 1090 M liquid chromatograph with a diode-array UV detector, 3DR solvent delivery system and a Series 7994 A workstation. Solutes: 2,6-diaminoanthraquinone (2,6-DAAQ), 1,2-diaminoanthraquinone (1,2-DAAQ), 2-aminoathraquinone (2-AAQ), 1-aminoanthraquinone (1-AAQ) and anthraquinone (AQ). Column: 300 × 4.2 mm Silasorb SPH C_{18}, 7.5 μm; flow-rate, 1 ml/min; detection, UV at 260 min; linear gradient of 1,4-dioxane in water: (top) from 35 to 100% in 10 min, (bottom) from 40 to 100% in 15 min.

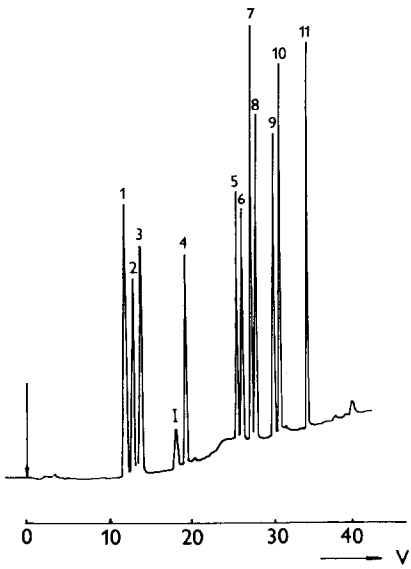

Fig. 3. Optimized reversed-phase separation of a mixture of eleven phenylurea herbicides using a linear gradient of methanol in water: $\varphi = 0.25 + 0.01749V$ (φ = concentration of methanol, vol-% · 10^{-2}; V = volume of the eluate, ml). Column: Silasorb SPH C_{18}, 7.5 μm, 300 × 4.1 mm I.D.; flow-rate, 1 ml/min; detection, UV (254 nm). Solutes: 1 = hydroxymetoxuron; 2 = desphenuron; 3 = phenuron; 4 = metoxuron; 5 = fluometuron; 6 = chlortoluron; 7 = isoproturon; 8 = diuron; 9 = linuron; 10 = chlorbromuron; 11 = neburon.

Another example is the optimization of the reversed-phase separation of a mixture of eleven phenylurea pesticides and related compounds on a 300 × 4.2 mm I.D. Silasorb C_{18} column using a linear gradient of methanol in water. The gradient parameters were optimized for the gradient volume V_G = 45 ml and φ_G = 100% methanol (Fig. 3)[12]. Here, the separation of the solute pairs hydroxymetoxuron–desphenuron–phenuron, fluometuron–chlortoluron, isoproturon–diuron and linuron–chlorbromuron presents some problems. From the predicted resolution map, the optimum gradient was selected, starting at 25% methanol with 1.75% increase per 1 ml of eluate, which allowed a maximized minimum resolution of about 1.2 to be achieved.

In comparison with the "critical" pair optimization method, direct calculation of the optimum gradient slope and initial mobile phase composition is not possible, but the optimum values of these parameters are determined from the plot (or table) of resolutions of all the solute pairs with adjacent bands predicted by computer calculations, which makes the "resolution mapping" method more suitable for the optimization of the gradient profile for the separation of complex mixtures, where more than a single pair of solutes are likely to be difficult to resolve. The main advantage of the "critical pair" optimization method for less complex sample mixtures is that it is not necessary to determine experimentally the constants of the log k' *versus* φ dependences for more than three compounds, namely for the "critical" pair, j and k, and the compound i the elution volume of which should be minimized. This advantage is of course lost if more (or all) solutes from the sample mixture are tested as the compound i.

Another possibility for the predictive optimization of gradient elution is just to try various combinations of the parameters A, B and κ to calculate simulated chromatograms with the aid of a computer, using eqns. 6 and 7 or 11 and 12, until satisfactory results are achieved. For example, Baba *et al.*[41–43] applied this approach successfully with the gradient function 10 and eqns. 11 and 12, introduced[17] for the predictive optimization of the anion-exchange chromatography of inorganic polyphosphates and oligonucleotides using a convex concentration gradient of potassium chloride in water with addition of 0.1% sodium ethylenediaminotetraacetate or linear and convex concentration gradients of a phosphate buffer in 20% aqueous acetonitrile. It would also be possible to use predictive calculations of elution volumes, band widths and resolution in gradient elution chromatography instead of real experiments in connection with the computer-assisted statistical multiparameter simplex optimization.

Optimization of binary "step" gradients

Stepwise gradient elution employs a set of subsequent isocratic steps with a gradually increasing concentration of the more efficient eluting component in the mobile phase. Our optimization approach is simpler than the similar approach proposed by Borówko *et al.*[44,45]. It is based on the equations for elution volumes and band widths derived for this type of elution[9,17]. The main objective of the computer-assisted optimization is to calculate the volume of the mobile phase, V_{ei}, and the concentration of the more efficient eluting component φ_i in each of the subsequent steps i, so as to accomplish the elution of each sample compound in a separate step, while the resolution of all the sample compounds should be kept at a desired level, R_{gd}[9]. The order of elution should be known and the calculation procedure starts with

step 1, where the two least retained solutes should be eluted under isocratic conditions. Further details of the optimization approach are given in Appendix 2.

This optimization strategy has been also slightly adapted to the construction of optimized step gradients, where two sample solutes instead of a single one are eluted in each step[9].

The basic condition for the successful application of this optimization approach is that R_{gd} can be achieved in a given system. Sometimes the concentration φ_n calculated using this method for the elution of a solute in step n is lower than the concentration in the preceding step $n-1$, $\varphi_{(n-1)}$, or even the calculated value of the elution volume $V_{R(n)}$, of the solute supposed to be eluted in the step n may sometimes be lower than the sum of the volume of the eluate in all the preceding steps from 1 to $n-1$. This means that the concentration $\varphi_{(n-1)}$ is too high and in this instance the calculation should be repeated using the predicted value φ_n in step $n-1$ instead of $\varphi_{(n-1)}$. The elution of two compounds originally intended in two different steps, $n-1$ and n, is now accomplished in a single step, $n-1$.

The optimization method for stepwise gradient elution was verified on the reversed-phase separation of a mixture of barbiturates, using a step gradient of methanol in water, optimized so as to achieve desired resolution $R_{sd} = 1.75$ for the solutes in the sample mixture[9].

If the individual gradient steps are narrow and if the concentration change between the individual steps is too great, distorted or split peaks of some solutes may appear, which are difficult to interpret and quantitate. For this reason, too narrow steps should be avoided.

This procedure can also be modified for segmented gradients consisting of several subsequent linear segments, as outlined in ref. 1, p. 135. Recently, Ghrist and Snyder[39,40] suggested an alternative approach for optimizing linear segmented gradients. In contrast to our method, in which the duration and composition of the mobile phase (or linear gradient profile) in each segment are "tailor- made", optimized for the best separation of subsequently eluted sample compounds, their approach relies on a trial-and-error strategy, using predictive computer simulations, in which the influence of preceding segments on the elution of a sample solute in the following segment is neglected to a first approximation. Here again, the gradient steepness, range and duration of each segment are optimized subsequently, starting with the first segment. For the optimization, the sample solutes are either divided into several groups, yielding closely spaced "clusters" of bands in the chromatogram, or the number of "critical" pairs (or groups) of solutes is known. The number of "clusters" or "critical" groups determines the number of segments in the gradient. The steepness, time and range of the gradient in the first segment are optimized for the least retained "cluster" or "critical" group of sample solutes, using the approach suggested by Snyder and co-workers[27,35–37] for simple continuous binary gradients. The same procedure is repeated for the second "cluster" or "critical" group of sample compounds, following the elution of the first group, to optimize the gradient steepness and range in the second segment, neglecting the possible influence of the preceding segment on the separation. This influence is subsequently compensated for by trial-and-error tuning of the gradient steepness in the second segment and, if necessary, also in the first segment. When appropriate, the same procedure is repeated for a third, fourth, etc., segment[40].

OPTIMIZATION OF TERNARY SOLVENT GRADIENTS

Ternary solvent gradients should be used only if the selectivity in binary mobile phases is not adequate for a good separation of all important sample solutes. These gradients are much more difficult to interpret and to optimize than binary gradients.

Full optimization of a ternary gradient would involve the shape, slope and initial concentrations of the partial gradients of the two stronger eluting components X and Y in the mobile phase, *i.e.*, six parameters should be optimized simultaneously. Although this is theoretically possible using the simplex method in a six-dimensional (or with a pre-set gradient shape in a four-dimensional) space, the number of experiments necessary for such an optimization would be enormous. Even with simulations of chromatograms by a computer using predictive calculations from eqns. 15 and 16, several hundred thousand simulation calculations for each solute pair would be necessary to cover the full space of the optimized parameters, including the curvature of a gradient, and more than 10 000 for the calculation of the retention data with a pre-set gradient shape. Although it would be possible to sort these data and to find the combination of the initial concentrations A_x, A_y and slopes B_x, B_y for the partial gradient profiles of the two stronger eluting solvents using a modern computer, this seems inpractical as most of the calculation effort would be wasted when "scouting" for the combinations of the gradient parameters that are not likely to yield satisfactory results. For this reason, a rational limitation of the number of parameters to be optimized seems useful. Such a limitation should take into account the nature of the separation problem, which will dictate the type of ternary gradient and the optimizaton strategy.

A generally complex response surface in ternary gradient elution liquid chromatography has led some workers to the conclusion that the optimization of ternary gradients is almost impossible. One exception is the application of the extended Sentinel optimization method to so-called "iso-selective multi-solvent gradients"[2,40], mentioned in the Introduction. Here, the optimization is limited to the concentration ratio of the individual solvents and to the time and slope of the gradient.

We have proposed predictive optimization methods for various specific types of ternary gradients in reversed-phase chromatography, based on computer-assisted predictive calculations of the elution volumes, band widths and resolution of sample solutes from the retention data acquired in binary mobile phases, using eqns. 15–17.

Ternary solvent gradients may be classified into three basic types: "solvent strength", "selectivity" and "combined selectivity–solvent strength" ternary gradients.

(1) Ternary "solvent strength" gradients make use of a pre-set concentration ratio of two organic solvents X and Y in aqueous mobile phases, $g = \varphi_x : \varphi_y$, which is kept constant during the gradient elution, while the elution strength is increased by increasing the sum of the concentrations, $\varphi_T = \varphi_x + \varphi_y$, in a linear manner. Although the separation selectivity also may change to a certain extent in the course of such a gradient, the separation is primarily affected by changes in the absolute retention of sample solutes. These gradients are relatively the most straightforward to be used and are suitable for sample mixtures with a wide retention range, for which an adequate separation selectivity cannot be achieved in binary mobile phases, but it may be attained at an optimized solvent ratio in a ternary mobile phase under isocratic

conditions. Here, the main objective of gradient elution is to speed up the elution of strongly retained compounds.

Ternary "solvent strength" gradients are controlled by a single gradient function:

$$\varphi_T = A + BV \tag{21}$$

As is derived in Appendix 3, the solute capacity factors depend on φ_T as follows[13]:

$$\log k' = a_T - m_T\varphi_T = a_T - m_TA - m_TBV \tag{22}$$

Eqn. 22 is formally identical with eqn. 5, wich means that the same expressions (eqns. 6 and 7) as in binary gradient elution can be employed for the predictive calculations of V_g and w_g, with $a = a_T$ and $m = m_T$. This means that once the ratio g has been adjusted, the optimization of a ternary "solvent strength" gradient can be performed in the same way as with binary gradients, by either the "critical" pair or "resolution mapping" method. In contrast to binary gradients, the parameters a_T and m_T of sample solutes are adjusted by the choice of the concentration ratio, g, of the two organic solvents in the mobile phase.

(2) In ternary "selectivity" gradients, the sum of concentrations of the organic solvents X and Y, $\varphi_T = \varphi_x + \varphi_y$, is kept constant during the gradient elution, but the ratio of concentrations, $g = \varphi_x : \varphi_y$, is changed with time. To hold φ_T constant, an increase in φ_x should be compensated for by an equivalent decrease in φ_y per unit volume of the eluate. This means that $B_x = -B_y = B$ and $A_x + A_y = \varphi_T$. During such gradients, the elution strength also changes to some extent (increases if X is a stronger eluent than Y and decreases in the opposite case), but the primary factor affecting the separation is the selectivity change.

The objective of ternary "selectivity" gradients is to improve the separation selectivity rather than to speed up the elution of strongly retained compounds. Such gradients can be applied to sample mixtures with a limited retention range, for which neither binary nor ternary mobile phases yield a satisfactory isocratic separation. If in such a sample mixture a pair or a group of relatively weakly retained compounds show a good separation selectivity in binary mobile phases solvent Y–water, but a poor selectivity in binary mobile phases solvent X–water and at the same time the opposite applies to another pair or group of sample solutes with a relatively stronger retention, a "selectivity" gradient may improve the overall separation in the chromatogram.

As is shown in Appendix 4, a simple equation can be derived for the dependence of the solute capacity factors on the volume of eluate, V, from the start of a ternary "selectivity" gradient, formally identical with eqn. 5 for binary solvent gradients, where the parameters a, m and A depend on the sum of the concentrations of X and Y, φ_T, and on the initial ratio of these two concentrations at the start of the gradient, $g_0 = A_x : A_y$. Consequently, eqns. 6 and 7 can be applied to calculations of the elution volumes and band widths in a ternary "selectivity" gradient, after appropriate substitution for a, m and A (see Appendix 4)[13].

Ternary "selectivity" gradients can be optimized using either the "critical" pair or the "resolution mapping" method. In contrast to the simultaneous optimization of A and B for binary gradients the slope B and the initial concentration ratio g_0 are optimized simultaneously.

(3) In "combined selectivity–solvent strength" ternary gradients, both the sum and the ratio of concentrations of the two stronger eluting agents X and Y are changed with time. In this instance, the elution volumes of sample solutes should be calculated from eqns. 15 and 16 using an iterative method, as the gradient parameters A_x, A_y, B_x and B_y are not interrelated.

This general type of ternary gradient is most difficult to optimize. It may be useful for the separation of sample mixtures with a wide retention range, where a group of weakly retained compounds cannot be resolved in binary mobile phases X–water and another group of strongly retained sample solutes fails to be separated in binary mobile phases Y–water. A selectivity gradient of simultaneously increasing φ_x and decreasing φ_y could possibly yield an adequate separation of all sample solutes, but in too long a time. Hence the rate of increase of φ_x should be greater than the rate of decrease of φ_y, which means that $\varphi_T = \varphi_x + \varphi_y$ should increase with time.

To optimize this type of linear ternary gradient, we have suggested a simplified strategy[11]. A gradient of increasing φ_x is designed first in order to achieve the separation of the group of strongly retained compounds in as short a time as possible, using the same procedure as for binary gradients. Subsequently, a gradient of decreasing φ_y from an initial value A_y to zero is superimposed on the gradient of increasing φ_x. A_y and simultaneously $B_y = -A_y/V_G$ are optimized by calculating the elution volumes and resolution for the individual sample solutes at different A_y, using eqns. 15, 16, 13 and 17. From the dependence of resolution of the individual pairs of sample compounds with adjacent bands on A_y, the optimum values of A_y and B_y are found using the "resolution mapping" method so as to maximize minimum resolution in the sample mixture, as in the optimization of binary gradients.

This predictive optimization strategy has been verified for the reversed-phase separation of a mixture of nine phenolic compounds using ternary methanol–acetonitrile–water gradients. The last eluted compounds were not separated in methanol–water mobile phases and the second and third solutes were poorly separated in acetonitrile–water mobile phases. This applied also to binary solvent gradients and an adequate separation could not be achieved even using a "solvent strength" ternary gradient with a pre-selected concentration ratio of methanol and acetonitrile in water. To optimize the separation, the gradient of increasing methanol concentration was designed first. Superimposed on this gradient, a gradient of decreasing concentration of acetonitrile with an optimum initial concentration was selected from the dependence of R_g of the individual compounds on A_y. With the optimized ternary gradient acetonitrile–methanol–water from 20:0:80 to 0:100:0 in 60 min, all the sample phenolic compounds were successfully separated in less than 40 ml of eluate, which allowed V_G to be set at 40 ml for the optimized gradient[11].

The separation of twelve phenylurea herbicides using acetonitrile–methanol–water ternary gradients in reversed-phase systems may be given as another optimization example[47]. In the sample mixture, the separation of monolinuron from chlortoluron is better in acetonitrile–water than in methanol–water mobile phases, whereas the opposite applies to the pair hydroxymetoxuron–desphenuron (Fig. 4). The group of four solutes chlortoluron, monolinuron, metobromuron and diuron presents a difficult separation problem and the elution order in this group of compounds depends not only on the concentration ratio of methanol and acetonitrile, but also on the sum of concentrations of these solvents in a ternary mobile phase.

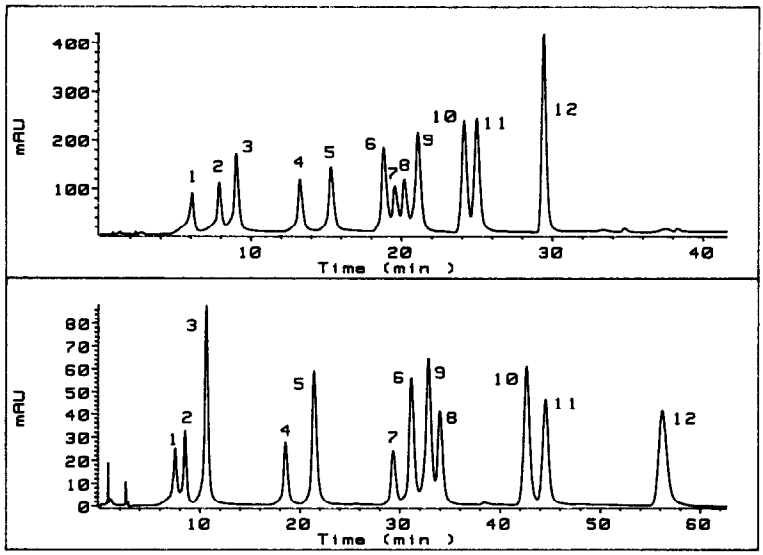

Fig. 4. Reversed-phase separation of a mixture of twelve phenylurea herbicides using linear gradients of methanol and acetonitrile in water. Column: Silasorb SPH C_{18}, 7.5 μm, (300 × 4.2 mm I.D.); flow-rate, 1 ml/min; instrument as in Fig. 2, operated at 230 nm. Solutes: 1 = hydroxymetoxuron; 2 = desphenuron; 3 = phenuron; 4 = metoxuron; 5 = monuron; 6 = monolinuron; 7 = chlortoluron; 8 = metabromuron; 9 = diuron; 10 = linuron; 11 = chlorbromuron; 12 = neburon. (Top) 25 75% methanol in water in 30 min; (bottom) 15–55% acetonitrile in water in 60 min.

Neburon is strongly retained and gradient elution is necessary to accomplish its elution in a reasonable time.

To optimize the separation of this sample mixture, the map of separation selectivity (*i.e.*, relative retention, α) of the solutes with adjacent bands was constructed as a function of the concentrations of acetonitrile and methanol in ternary mobile phases under isocratic conditions. It was found that the concentration ratio of methanol to acetonitrile, g, yielding the maximized minimum α is shifted to higher values with increasing sum of concentrations of the organic solvents, φ_T. At the time of elution of the group of four phenylureas most difficult to separate, the instantaneous φ_T is about 55—65%, which corresponds to an optimum g value of about 3–4. A ternary "solvent strength" gradient was optimized so as to maximize the separation of monolinuron and chlortoluron at $g = 4$ and yielded a separation improved with respect to methanol water and acetonitrile–water binary gradients (Fig. 5A).

A ternary "selectivity" gradient was then optimized for maximized resolution of the group of four most difficult to separate phenylureas within the gradient volume $V_G = 45$ ml. Under the predicted optimum conditions, the separation of the group of monolinuron, chlortoluron, metobromuron and diuron was better than that with the optimized "solvent strength" gradient, at the price of a slightly impaired resolution of desphenuron from phenuron, but the elution of neburon could not be accomplished within this gradient volume. A steeper gradient of acetonitrile after the end of the optimized "selectivity" gradient should be used for this purpose (Fig. 5B).

Finally, a combined "selectivity solvent strength" gradient was optimized in order to find the best initial concentration of methanol decreasing to the final

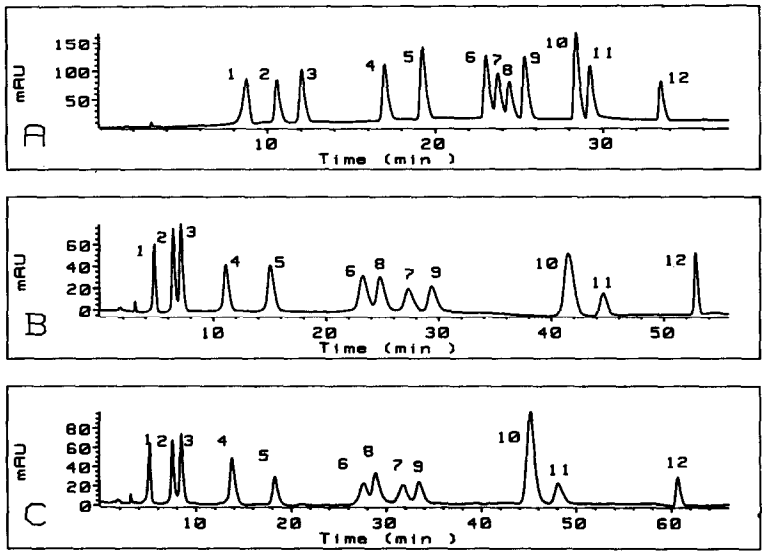

Fig. 5. Optimized reversed-phase separation of a mixture of twelve phenylurea herbicides using (A) "solvent strength", (B) "selectivity" and (C) "combined solvent strength–selectivity" ternary gradients of methanol and acetonitrile in water. Gradient profiles: (A) acetonitrile–methanol–water 5:20:75 to 20:80:0 in 45 min; (B) acetonitrile–methanol–water 0:40:60 to 40:0:60 in 45 min, then to 80:0:20 in 15 min; (C) acetonitrile–methanol–water 0:35:65 to 40:0:60 in 45 min, then to 60:0:40 in 25 min. Other conditions as in Fig. 4. Solutes: 1 = hydroxymetoxuron; 2 = desphenuron; 3 = phenuron; 4 = metoxuron; 5 = monuron; 6 = monolinuron; 7 = metobromuron; 8 = chlortoluron; 9 = diuron; 10 = linuron; 11 = chlorbromuron; 12 = neburon.

concentration of 0, 10, 20 and 30% in 45 min, while the concentration of acetonitrile increased linearly from 0 to 40% in the same time (V_G = 45 ml). The gradient optimized in this way was similar to the optimized "selectivity" gradient and the separation achieved is shown in Fig. 5C. The elution of neburon is now accomplished in a similar time to that with the "selectivity" gradient, but the separation of the four "critical" solutes is poorer than with the optimized "selectivity" gradient[47].

Possibly, with other combinations of V_G and initial and final concentrations of acetonitrile, a better optimized ternary gradient could be arrived at, but at the cost of a great number of optimization calculations. In this instance, the optimized "selectivity" ternary gradient followed by a steeper elution strength gradient to speed up the elution of the most strongly retained compound, neburon, made it possible to develop an acceptable separation with much less calculation effort.

LIMITATIONS OF PRESENT OPTIMIZATON METHODS AND POSSIBILITIES OF TRANSFERRING OPTIMIZED GRADIENTS TO OTHER COLUMNS

The optimization methods for binary and ternary gradients discussed here do not provide full optimization of all gradient parameters. Rather, the optimization strategy is focused on the parameters that are most likely to affect the quality of separation most significantly. The choice of the optimization strategy depends on the specific character of the separation problem, which should be considered by user. The

type of problem can be identified from a few preliminary experiments necessary for the acquisition of the parameters of $k' = f(\varphi)$ equations for the individual solutes. The reason for this optimization strategy is to limit the effort necessary for a large number of experiments or predictive calculations connected with full optimization of all the parameters, which would be very tedious, especially for ternary gradients.

There are some limitations to the optimization methods discussed here. First, not all the instruments can reproduce accurately a preset gradient profile[1,48]. The effect of the instrumentation on the actual gradient profile and on the agreement between the experimental and predicted retention data has been investigated by Jandera and co-workers[1,48,49], Quarry *et al.*[50,51] and Ghrist *et al.*[52]. If we disregard possible pump failure to deliver the preset flow-rate of the mobile phase or its components because of occasional effects leading to an improper functioning of the pump check valves (air bubbles, dirt, etc.)[52], the principal possible sources of distortion of a pre-set gradient profile are as follows:

(a) the two pumps used to produce the gradient in a high-pressure part of the instrument may not have exactly the same performance characteristics[1,48];

(b) the working cycle of the solenoid check valves used in some gradient instruments to mix the individual solvents at a low pressure is not well synchronized with the fill-and-deliver cycle of a high-pressure pump to which the pre-mixed mobile phase is introduced[1];

(c) volume changes connected with the mixing of the mobile phase components are not adequately compensated for[48];

(d) imperfect mixing of the mobile phase components and large inner volumes in the solvent delivery systems may lead to a change of the programmed gradient profile or to an important gradient delay[48,50–52]. With most well designed modern HPLC equipment, the instrumental limitations are not very critical. If the gradient delay is significant and cannot be neglected, it can be compensated for either by delaying the sample injection with respect to the start of the gradient, or by using the appropriate equations for calculations of V_g and w_g, respecting the gradient delay (ref. 1, p. 137, and refs. 50–52);

(e) change in column retention characteristics with time when the column is used over a longer period and differences between the chromatographic properties of the individual batches of the column packing material[46].

In normal-phase chromatography on polar adsorbents, the gradient profile may sometimes be distorted by a preferrential adsorption of the more polar organic solvent from the mobile phase on to the surface of the adsorbent with resulting shifts in elution volumes, distortion of peak shapes or even band splitting and the occurrence of "ghost" peaks in the chromatogram[49,51]. This effect can be minimized if the column is pre-equilibrated with the polar solvent, the initial concentration of which should be at least 5–10% at the start of the gradient[1].

The predictive optimization methods are also affected by simplifications involved in the calculation of the retention characteristics, mainly by a limited retention range of validity of simple k' *versus* φ equations, such as eqns. 3 and 9[1,52]. Nevertheless, the accuracy of predictive calculations for binary gradients is usually better than 5% relative; the calculations of elution volumes in the systems with ternary gradients may be subject to greater errors. The impact on the results of optimization is diminished by the fact that the relative retention is affected by these errors less than the absolute values of the elution volumes.

The optimized gradient conditions apply only for a given column packing used for the acquisition of the parameters of $k' = f(\varphi)$ equations. They can usually be used directly for another column packed with the same material, provided that there are equal column lengths and diameters and equal flow-rates of the mobile phase. In gradient elution chromatography, the predicted elution volumes and optimized separation conditions can be transferred to another column or flow-rate of the mobile phase only under the condition that the product BV_m is constant[1]. Then the solute elution volume V_{gII} expected on a column II of length L_{II}, diameter d_{II} and dead volume V_{mII} may be calculated from the elution volume of this solute, V_{gI}, obtained on a column I of the length L_I, diameter d_I and dead volume V_{mI} as follows[1]:

$$V'_{gII} = V'_{gI} \cdot \frac{V_{mII}}{V_{mI}} = V'_{gI} \cdot \frac{L_{II}}{L_I} \left(\frac{d_{II}}{d_I}\right)^2 \tag{23}$$

The predicted optimum gradient may sometimes require some additional experimental fine tuning, but generally the calculated profile of the gradient is close to the objective of optimization and the number of experiments necessary for optimization is significantly lower than in the empirical or statistical optimization methods.

APPENDIX 1

Optimization scheme for a binary gradient using the "critical" pair method

First, the gradient shape, κ, is preset, then the sample compounds j and k representing the "critical" pair are selected and another solute, i, the retention of which should be minimized, is chosen. The interval of practically possible values of A is defined: $A > 0$ and $A < A_{max}$, where A_{max} is the concentration of the more efficient eluting component in the mobile phase at which the resolution desired, R_{gd}, is just achieved under isocratic conditions. A_{max} is calculated from the well known equation for resolution, R_s, applying under isocratic conditions:

$$R_S = 2 \cdot \frac{V_{Rk} - V_{Rj}}{w_j + w_k} = \frac{\sqrt{N}}{2} \cdot \frac{k'_k - k'_j}{k'_j + k'_k + 2} \tag{A1}$$

after setting $R_s = R_{gd}$, and introducing the appropriate $k' = f(\varphi)$ functions applying in a given chromatographic system into eqn. A1, with the parameters a and m determined for compounds j and k in preliminary isocratic experiments (*e.g.*, eqn. 3 or 9).

The interval of A is then subsequently narrowed as follows. As eqn. A1 does not apply under gradient conditions (k'_k and k'_j are not constant), the resolution in gradient elution chromatography should be calculated after introducing the expressions for V_g and w_g (*e.g.*, eqns. 6 and 7 for reversed-phase systems or eqns. 11 and 12 for normal-phase and ion-exchange systems) into the definition equation

$$R_{sg} = 2 \cdot \frac{V_{gk} - V_{gj}}{w_{gk} + w_{gj}} \tag{A2}$$

After setting $R_{sg} = R_{gd}$, eqn. A2 can be solved to calculate the gradient slope B corresponding to $A = 0$, $A = A_{max}$ and $A = 0.5A_{max}$. The elution volumes V_{gi} are calculated for each of the three A values and that of the interval limits $A = 0$ or $A = A_{max}$, which yields higher value of V_{gi} is rejected. The initial interval of the A values is halved in this way, either to the limits from 0 to $0.5A_{max}$, or from $0.5A_{max}$ to A_{max}. The values of B and V_{gi} are calculated using the same procedure for the value of A from the centre of the new interval. V_{gi} corresponding to the limits of the new interval of A values are again compared and then the interval is halved to one quarter of the initial interval. The whole procedure is repeated until a minimum value of V_{gi} is found with a preset precision of the calculated parameters A and B of the optimized gradient profile. The detailed scheme of the program used for this predictive optimization approach can be found in ref. 8.

APPENDIX 2

Optimization scheme for a binary "step" gradient

The optimization of a binary step gradient starts with step 1, where the concentration φ_1 of the stronger eluent is calculated from eqn. A1 after introducing the appropriate $k' = f(\varphi)$ functions (*e.g.*, eqn. 3 or 9) and the preliminarily determined parameters a and m for the two least retained solutes, setting $R_s = R_{gd}$ so as to achieve just the desired resolution R_{gd} for this pair of compounds. Then the capacity factors for more retained compounds eluted in each of the subsequent steps n, k'_n, which are necessary to achieve R_{gd}, are subsequently calculated from eqn. A3, starting with the step 2 ($n = 2$):

$$k'_n = \frac{V'_{R(n-1)} - \sum_{i=1}^{n-1} V_{ei} + \frac{2V_m R_{gd}}{\sqrt{N}} [k'_{(n-1)} + 2]}{V_m - \sum_{i=1}^{n-1} \frac{V_{ei}}{k'_i} - \frac{2V_m R_{gd}}{\sqrt{N}}} \qquad \text{(A3)}$$

where $V'_{R(n-1)}$ and $k'_{(n-1)}$ are the net elution volume and the capacity factor of the last eluted compound in step $(n-1)$, k'_i is the capacity factor of the solute eluted in the step $i<n$ and V_m and N are the column dead volume and plate number (isocratic). The value of k'_n calculated from eqn. A3 is used to determine V_{ei} in the nth step, V_{en}:

$$V_{en} = V'_{R(n)} + \frac{2V_m \cdot R_{gd}}{\sqrt{N}} (k'_n + 1) - \sum_{i=1}^{n-1} V_{ei} \qquad \text{(A4)}$$

where $V'_{R(n)}$ is the net elution volume of the solute eluted in step n:

$$V'_{R(n)} = V'_{R(n-1)} + \frac{2V_m R_{gd}}{\sqrt{N}} [k'_n + k'_{(n-1)} + 2] \qquad \text{(A5)}$$

The concentration φ_n of the more efficient eluting component in step n is calculated after introduction of the value of k'_n into the appropriate equation for the $k' = f(\varphi)$

function applying in a given chromatographic system. This calculation scheme is repeated for each step until the conditions for elution of the last sample compound are determined[9].

APPENDIX 3

Derivation of equation of k' versus φ_T and predictive optimization strategy for ternary "solvent strength" gradients

After combination of the two basic conditions for this type of gradient:

$$g = \varphi_x : \varphi_y = A_x : A_y = \text{constant} \tag{A6}$$

and

$$\varphi_T = \varphi_x + \varphi_y \tag{A7}$$

we obtain

$$\varphi_x = \varphi_T \cdot \frac{g}{1 + g} \tag{A8}$$

$$\varphi_y = \varphi_T \cdot \frac{1}{1 + g} \tag{A9}$$

After combination with eqn. 13, the following relationship between k' and φ_T results:

$$\log k' = \frac{a_x g + a_y}{1 + g} - \frac{m_x g + m_y}{1 + g} \cdot \varphi_T = a_T - m_T \varphi_T \tag{A10}$$

which is formally identical with eqn. 5 for binay gradients.

To optimize a "solvent strength" gradient, the same methods can be used as for binary gradients but the concentration ratio g should first be adjusted. The ratio g may be optimized as the concentration ratio of the two organic solvents in ternary mobile phases under isocratic conditions, *i.e.*, on the basis of k' of the sample solutes calculated from eqn. 13 with the parameters a_x, m_x and a_y, m_y determined in binary water–organic solvent X and water–organic solvent Y mobile phases, respectively[33]. From the predicted capacity factors, V_R, w, α or R_s are calculated for ternary mobile phases with varous ratios g and "resolution maps" are constructed, from which the optimum g is selected for maximized minimum resolution or for maximized separation selectivity in the sample mixture[33].

APPENDIX 4

Derivation of equation of k' versus φ_T and g_0 and predictive optimization strategy for ternary "selectivity" gradients

The two basic conditions for a "selectivity" gradient:

$$\varphi_T = A_x + A_y = \text{constant} \tag{A11}$$

and

$$B_x = -B_y = B \tag{A12}$$

can be combined with eqns. 13 and 14 to yield

$$\log k' = \frac{a_x g_0 + a_y - (g_0 m_x + m_y)\varphi_T}{1 + g_0} + \left(\frac{a_x - a_y}{\varphi_T} - m_x + m_y\right) BV$$

$$= a - mA - mBV \tag{A13}$$

where $g_0 = A_x : A_y$ is the ratio g at the start of the gradient elution,

$$A = -\frac{\varphi_T}{1 + g_0} \tag{A14}$$

$$B = B_x = -B_y \tag{A15}$$

$$a = a_x - m_x\varphi_T \tag{A16}$$

$$m = \frac{a_y - a_x}{\varphi_T} + m_x - m_y \tag{A17}$$

The same strategy and predictive equations as with binary gradients can be used for the calculation of retention data and for the optimization of ternary "selectivity" gradients. Prior to this optimization, the sum of concentrations, φ_T, should be adjusted on the basis of the expected elution times of sample solutes, which can be estimated from the results of preliminary experiments with binary mobile phases, necessary for the determination of the parameters a_x, m_x and a_y, m_y. The "resolution mapping" method is especially suitable for "selectivity" gradients, because the gradient volume, V_G, can be easily adjusted as the elution volume of the last eluted compound in the binary mobile phase where the concentration of the less strong of the eluting agents X and Y is equal to φ_T. If the "selectivity" gradient is stopped at $\varphi_y = 0$, then the initial ratio g_0 determines the slope of the gradient:

$$B = \frac{\varphi_T}{V_G\,(1 + g_0)} \tag{A18}$$

The dependences of resolution for all the pairs of compounds with adjacent bands on g_0 can be constructed in either graphical or tabular form and g_0 for the maximized minimum resolution together with the appropriate gradient slope, B, are selected from these dependences to define the optimized gradient profile.

SYMBOLS

a, m, a_x, a_y, m_x, m_y	constants of the $k' = f(\varphi)$, $k' = f(\varphi_x)$, $k' = f(\varphi_y)$ functions
a_G	mean value of a_x and a_y during elution with a ternary gradient (eqn. 16)
a_T, m_T	a, m relating to the $k' = f(\varphi_T)$ function for "selectivity" ternary gradients
$g = \varphi_x{:}\varphi_y$	concentration ratio of stronger eluting components in elution with ternary gradients
g_0	g at the start of a "selectivity" ternary gradient
d	column diameter
k'	capacity factor of a sample solute
k'_a	k' at the start of gradient elution
k'_i, k'_n, $k'_{(n-1)}$	k' of a sample solute in steps i, n, $(n-1)$ in stepwise gradient elution
k'_f	instantaneous k' at the time of elution of the peak maximum in gradient elution chromatography
k'_j, k'_k	k' of sample solutes j, k under isocratic conditions
t_G	gradient time from the start to the end of a solvent programme
w	solute band width under isocratic conditions (in volume units)
w_g	w in gradient elution chromatography
w_j, w_k	w of sample solutes j, k
w_{gj}, w_{gk}	w_g of sample solutes j, k
A, B	initial concentration of a stronger eluting component and slope of the gradient (in concentration change per unit volume) for binary solvent gradients (eqn. 4)
A_x, B_x, A_y, B_y	A and B relating to partial linear gradients of the components X and Y for ternary solvent gradients
A_{max}	A necessary to achieve R_{gd} under isocratic conditions
B_1	first estimate of the gradient slope
F_m	volume flow-rate of the mobile phase
L	column length
N	column plate number (under isocratic conditions)
R_s	resolution of two sample solutes with adjacent bands under isocratic conditions
R_{sg}	R_s in gradient elution chromatography
R_{gd}	desired valuc of R_{sg}
V	volume of the eluate
V_g, V'_g	elution volume and net elution volume of a sample solute under gradient conditions
V_{gi}, V_{gj}, V_{gk}	V_g of sample solutes i, j, k
V_G	gradient volume from the start to the end of a solvent programme
V_{ei}, V_{en}	volume of the eluate in step i and in step n (last step) in stepwise gradient elution
V_m	column dead volume
V_{Rj}, V_{Rk}	elution volume of sample solutes j, k (isocratic)

$V'_{R(n)}$, $V'_{R(n-1)}$	net elution volumes of sample solutes eluted in step n and in step $(n-1)$ in stepwise gradient elution chromatography
$\alpha = k'_k/k'_j$	selectivity, *i.e.*, relative retention of solutes j and k under isocratic conditions
β	slope of a "linear solvent strength" gradient (eqn. 2)
κ	curvature parameter, characterizing the shape of a gradient (eqn. 10)
φ	volume concentration of the stronger eluting component in a binary mobile phase
φ_G	φ at the end of a gradient
φ_x, φ_y	φ of the stronger eluting components X and Y in a ternary mobile phase
$\varphi_T = \varphi_x + \varphi_y$	total concentration of the stronger eluting components X and Y in a ternary mobile phase

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