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Implementation and use of gradient predictions for optimization of reversed-phase liquid chromatography of peptides

Practical considerations

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ABSTRACT

The options in the implementation of gradient theory for optimization work are critically reviewed and evaluated for the case of the reversed-phase liquid chromatography of peptides. Various models are covered together with methods for the determination of model parameters. Approaches for calculating retention times and band widths from experimental data are discussed. Different kinds of extrapolation are compared with interpolation. This study was aimed at finding the best compromise between number of experiments, accuracy of predictions and simplicity of calculations. Implementation and the use of gradient predictions can be simple, and practical recommendations are given.

INTRODUCTION

The retention of peptides in reversed-phase liquid chromatography is extremely sensitive to the concentration of organic modifier in the mobile phase [1–5]. It is unusual that a peptide sample can be separated with a reasonable range of retention by isocratic elution. Separation of peptides by reversed-phase liquid chromatography is performed almost exclusively by gradient elution.

The theory of gradient elution is well established [6–8]. From the theory it is clear that the selectivity may vary with gradient slope. This is also commonly the case for peptides and proteins [4,9,10], and the general rule of thumb, that the resolution between all peaks will increase with increased gradient time, is not valid. Retention predictions based on gradient theory therefore emerges as a powerful tool for optimization in the reversed-phase chromatography of peptides, requiring few experiments.

Predictions based on gradient theory are generally highly accurate. In this work the relevant options that exist in the implementation and application of gradient theory are reviewed and evaluated. Four aspects are considered: the accuracy of predictions, the complexity of calculations, the necessary knowledge of the sample and the chromatographic system and the amount of experimental work necessary for making predictions. Gradient theory is regarded here as a tool for optimization of the gradient profile, hence only the accuracy of the predictions is studied and not that of the model parameters themselves.

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The various formulations of gradient theory that have been published are critically examined. Practical recommendations for the implementation of gradient predictions for "real samples" are presented and discussed. The implementation and use of these predictions can be simple and this work will hopefully encourage more peptide chromatographers to use gradient theory.

THEORY

A complete presentation of gradient theory can be found in publications by Jandera and Churáček [8] and Snyder and Stadalius [6,7]. Only the final expressions and the underlying assumptions will be presented in this paper. The notation of Jandera and Churáček has mainly been followed and the symbols are listed at the end of the paper.

Retention volume

Most expressions of retention volume in gradient elution in reversed-phase liquid chromatography are based on a linear relationship between k' and concentration of organic modifier for isocratic elution:

$$\log k' = a - m\varphi \tag{1}$$

From theory based on various models of physical chemistry of chromatography it has been argued that a quadratic model is a more correct description [11,12], and non-linear relationships have also been observed [12–15]. Thorough discussions have resulted in the general opinion that the function is non-linear but can be approximated by a linear function in k' range where most of the migration occurs ($1 < k' < 10$) [13,15–18]. Several workers have also found that they can obtain accurate predictions of retention for peptides and amino acids using expressions based on this assumption [2,19–23]. From a practical point of view, a linear function is advantageous as calculations based on a non-linear model are complex and require more experiments [15,24–26].

Several attempts have been made to relate the parameters a and m to solute properties such as molecular mass and hydrophobicity, but with only limited success [2–4,7,27–29]. In addition, most samples contain solutes for which these properties are unknown, making this approach restricted in application. A correlation between a and m has been sought, again with mixed results [15,30]. In practice, a and m have to be determined experimentally for all solutes in a sample.

Several ways to describe a linear gradient have been given in the literature. The different terms "slope", denoted B, used by Jandera and Churáček, "steepness", b, used by Snyder's group, and "rate", s, can be confusing; see Table I for an explanation. This disparity has been pointed out and discussed by Jandera and Churáček [8,17]. These parameters are related to one another as follows:

$$b = V_m mB = V_m m(s/F) \tag{2}$$

In this paper, the parameter B will mainly be used and all gradients will be considered as linear:

$$\varphi = \varphi_0 + BV \tag{3}$$

On the basis of eqns. 1 and 3, the following expression for the retention volume can be derived [6,8]:

$$V_g = \frac{1}{mB} \cdot \log (2.3 V_m mBk'_0 + 1) + V_m \tag{4}$$

where k'_0 is k' at the starting concentration of organic modifier;

$$k'_0 = 10^{a - m\varphi_0} \tag{5}$$

The gradient is inevitably preceded by an isocratic step, because it takes some time for the gradient to reach the column. The following expression includes migration during the passage of the dwell volume [8,31]:

$$V_g = \frac{1}{mB} \cdot \log (2.3 V_{mg} mBk'_0 + 1) + V_m + V_d \tag{6}$$

where V_{mg} is the part of the dead volume where gradient elution occurs. V_{mg} is given by

$$V_{mg} = V_m - \frac{V_d}{k'_0} \tag{7}$$

The only assumption for k'_0 in eqn. 6 is that it should be larger than V_d/V_m, otherwise the solute

TABLE I

SIMILAR TERMS DESCRIBING LINEAR GRADIENT

Name	Symbol	Unit	Meaning/advantage/disadvantage
Rate	s	%/min	Change in concentration of organic modifier per unit time. Constant rate means constant gradient time if the starting and final concentration are fixed. Most chromatographers find this measure most natural. Selectivity, retention time and volume will change if the flow is altered, even if the rate is constant
Slope	B	%/ml	Change in concentration of organic modifier per unit volume. Constant slope gives constant selectivity and retention volume (but not retention time). A specified slope says nothing about gradient time
Steepness	b	–	Apparent slope, the "acceleration" of a solute as it migrates through the column. Band width and other separation characteristics are directly related to this, more fundamental, parameter. The steepness is solute dependent; it will not be the same for all solutes eluted with the same gradient. This parameter is also conceptually difficult

will reach the detector before the gradient. If the product $2.3V_{mg}mBk'_0$ is large, the following approximation can be made [2,7,9]

$$V_g = \frac{1}{mB} \cdot \log (2.3V_{mg}mBk'_0) + V_m + V_d \quad (8)$$

The latter expression has the benefit of simplifying the parameter fitting (see later description). Note that migration during the dwell volume is still acknowledged.

Band width

In isocratic elution, the band width is dependent on plate number, capacity factor and dead volume, leading to the following expression:

$$W_b = \frac{4V_m}{\sqrt{N}} \cdot (k' + 1) \quad (9)$$

In gradient elution it is generally assumed [6,32] that the band width is dependent on the capacity factor when the compound leaves the column, k'_f, apart from plate number and dead volume. This gives

$$W_{bg} = \frac{4V_m}{\sqrt{N}} \cdot (k'_f + 1) \quad (10)$$

where W_{bg} is the band broadening arising from the gradient elution. k'_f is given by [6]

$$k'_f = 1/[2.3V_m mB + 1/(k'_0)] \quad (11)$$

As the migration of the tail of the peak is faster than that of the centre, and *vice versa* for the front, bands will be compressed. A peak compression factor, G, has been introduced [33], giving rise to a slightly different expression for W_{bg}, namely

$$W_{bg} = \frac{4V_m}{\sqrt{N}} \cdot (k'_f + 1)G \quad (12)$$

An explicit expression for this peak compression has been derived [34]:

$$G^2 = \frac{1 + p + \frac{p^3}{3}}{(1+p)^3} \quad (13)$$

where the parameter p is given by

$$p = 2.3V_m mB \cdot \frac{k'_0}{k'_0 + 1} \quad (14)$$

In some papers, an expression based on the capacity factor when the solute has migrated half way through the column, $\bar{k}$, is used [1,35], leading to a different form of eqn. 10:

$$W_{bg} = \frac{4V_m}{\sqrt{N}} \cdot (\bar{k}/2 + 1) \quad (15)$$

Usually it is stated that [6,10,36]

$$\bar{k} = 1/1.15V_m mB \quad (16)$$

which is based on the assumption that $1/k_0'$ is much smaller than $1.15V_m mB$. The full expression is [7,37]

$$\bar{k} = 1/[1.15V_m mB + 1/(k_0')] \quad (17)$$

Hence eqns. 10 and 15 are equivalent when $1/k_0'$ is much smaller than $1.15V_m mB$, k_f' then becomes equal to $\bar{k}/2$ [36]. When this assumption is not valid, eqn. 10 should be used [7].

When $V_m mB$ is <1, eqn. 13 can be simplified [7]:

$$G = (1 + \bar{k})/(2 + \bar{k}) \quad (18)$$

In some papers, an expression for W_{bg} is made from eqns. 15 and 18, which lead to a simple form [1,35]:

$$W_{bg} = \frac{2V_m}{\sqrt{N}} \cdot (\bar{k} + 1) \quad (19)$$

Note that eqns. 15–19 are all various approximations of the more general expressions seen in eqns. 12–14.

It has been observed that the model for band width gives an underestimate at high values of $V_m mB$ [6,35,38]. Possible causes for this have been thoroughly discussed, without any satisfactory explanation [22]. To compensate for this deviation, an empirical correction factor, J, has been introduced [35]:

$$W_{bg} = J \cdot \frac{4V_m}{\sqrt{N}} \cdot (k_f' + 1)G \quad (20)$$

J has been presented as an empirical polynomial based on experimental data [35]:

$$J = 0.99 + 1.70(V_m mB) - 1.35(V_m mB)^2 + 0.48(V_m mB)^3 - 0.062(V_m mB)^4 \quad (21)$$

In later work by Dolan *et al.* [39], the following correction was used:

$$JG = 1.1 \quad (22)$$

Analogously to the retention volume, one can include the band broadening that takes place during the dwell volume. This leads to [8]

$$W_b = \sqrt{\frac{V_{mi}}{V_m} \cdot W_{bi}^2 + \frac{V_{mg}}{V_m} \cdot W_{bg}^2} \quad (23)$$

where V_m is replaced by V_{mg} in eqns. 11, 14, 20 and 21. W_{bi}, the isocratic band broadening in the starting mobile phase, is given by

$$W_{bi} = \frac{4V_{mi}}{\sqrt{N}} \cdot (k_0' + 1) \quad (24)$$

where V_{mi} is the part of the dead volume where migration occurs isocratically and is given by

$$V_{mi} = V_m - V_{mg} \quad (25)$$

The benefit of approximations

Long mathematical expressions are not practical as they obscure the basic relationships and are tedious to calculate manually. Approximations make long expressions short, hence their popularity. Today calculations are made by computers, and the need for approximations is smaller. Approximations are, however, still meaningful if they can speed up numerical methods, or even better, allow the calculations to be made by a non-iterative procedure. With the assumption of a linear relationship between log k' and φ (eqn. 1), the calculations of retention and band broadening from the parameters of the model are simple. The full expressions (eqns. 6 and 23) should therefore be used. The determination of a and m from gradient runs is more difficult, but with the assumption that $2.3V_{mg}mBk_0'$ is large it is simple (see later discussion). In contrast, there is no benefit in making an approximation for band width, as the plate number can be determined using eqn. 23, as will be shown below.

PARAMETER ESTIMATION

Dead volume, V_m

In the literature, there are various opinions on what exactly the dead volume is and how it should be determined experimentally [40–42]. A recent survey by Maliek and Jinno [43] revealed both controversy and confusion. Several comprehensive reviews have also been published (*e.g.*, ref. 44). In the gradient optimization, the method for determining V_m should be simple and rapid and result in accurate predictions, and does not necessarily have to give the "true" dead volume. It has also been shown that gradient

predictions are not very sensitive to errors in the dead volume [24]. The most common methods are (1) to measure the elution volume for a non-retained solute, "markers" (*e.g.*, refs. 45 and 46), (2) to measure the elution volume for a deuterated mobile phase component, typically D_2O (*e.g.*, refs. 42, 46 and 47) or (3) to measure the retention volume for a set of homologues and then do the determination by a parameter fit based on a thermodynamic model (*e.g.*, refs. 46–49). For practical reasons the first method is preferred, as a detector measuring refractive index is needed in the second method and the third method is too time consuming. The choice of non-retained solute is not obvious, and both inorganic salts and organic compounds have been suggested. Anions have the disadvantage of being excluded from parts of the mobile phase by negatively charged silanol groups, whereas cations are retained [45]. Neutral polar organic solutes seem more promising and in this work the often recommended uracil [44–46] was used.

It is also possible to determine the dead volume by data fitting, as a parameter in the model for retention volume. If the parameters a and m are unknown, which is the usual case, this means that at least three gradients have to be run. As the dead volume can be determined in a matter of minutes this is not a practical alternative, and it is better to use extra gradients for estimation of the dwell volume, as will be described below.

It has been indicated that the accessible volume is reduced if the size of the peptide is close to or larger than the pore size [2,37]. On the other hand, in the work by Larmann *et al.* [50], no difference in retention for large molecules was seen when columns with various pore sizes were used. This indicates that large molecules can reach all parts of the mobile phase in the column. By using columns with large pores (>100 Å) the potential difference is minimized [2,10]. In addition, large pores also promote high plate numbers [51–54] and recoveries of peptides [51,55]. It would also be difficult to determine individual dead volumes, so in practice the dead volumes must be assumed to be equal.

The dead volume, V_m, is dependent on the concentration of organic modifier [42,47,56]. In this work, V_m was determined at two concentrations of organic modifier for comparison. It has also been shown that the elution volume for the non-retained solutes is affected by flow-rate, temperature and amount of solute [45,46]. The determination of V_m should therefore be done at the same temperature and flow-rate as the calibration gradients. The influence of the amount of solute on the elution volume is not very pronounced for uracil [45], which is one reason for its popularity. To check this, the elution volume of uracil was determined with two different amounts of uracil, differing by a factor of 100.

A rough estimate of V_m can be made by assuming that V_m is a fixed proportion of the column volume [57]. This option was not evaluated as a meaningful test would have to involve several columns and also the gain in time is small, as V_m can be estimated within a few minutes.

Dwell volume, V_d

An incorrect estimate of the dwell volume is a common source of error in gradient predictions [21,25,57,58]. There are two experimental methods for estimation that can be regarded as reliable. One approach is to run a slow gradient of a UV-absorbing solution, without a column [27,58]. The dwell volume is then calculated as

$$V_d = V_{1/2} - \frac{1}{2} V_G \quad (26)$$

where $V_{1/2}$ is the volume when the absorbance has reached half its maximum value and V_G is the gradient volume. The other approach is to use more than two calibration gradients, with different slopes. The dwell volume is determined, together with the parameters a and m, by a fitting routine [22,23,58]. In this work the two methods are compared regarding the accuracy of absolute retention and difference in retention between two solutes.

Model parameters, a and m

The model parameters a and m can be determined from calibration gradients by some kind of parameter fitting. The key question is whether $\log (2.3 V_m m B k_0' + 1)$ can be approxi-

mated by log ($2.3V_m mBk'_0$), *i.e.*, if eqn. 8 can be used. The two cases lead to different parameter fitting routines, as described in a later section. In this work the parameter fitting was made for both cases, at different values of $2.3V_{mg}mBk'_0$ to establish when the approximation is valid.

Plate number, N

The plate number is usually determined for a small organic molecule, *e.g.*, naphthalene, eluted isocratically with $k' > 3$ with a high concentration of an organic modifier of low viscosity. The plate number that the manufacturer sends with a new column is typically determined in this way as the method gives a high estimate of the plate number. This method of estimating plate numbers will below be referred to as "the conventional isocratic method".

It is important to realise that the plate number is not a given constant for a specific column. It depends on several variables, of which the following are of interest in this context: diffusion rate of the individual components; viscosity of the mobile phase; capacity factor; and flow-rate.

It has been pointed out that the plate number generally is lower when working with peptides, at conventional flow-rates, than when working with small organic molecules [1,4]. The reason for this is the slow diffusion of macromolecules such as peptides [1,36]. The diffusion rate is related to the molecular mass and as peptides can have a broad range of molecular masses, the plate number will vary among peptides [4]. The diffusion rate is also dependent on the solvent. In the reversed-phase liquid chromatography of peptides, acetonitrile is the organic modifier most commonly used. The most popular alternative, 2-propanol [59–61], has a much higher viscosity, giving a lower plate number. The plate number is also related to k' [1,62], which is dependent on gradient slope and solute. In addition, the plate number is also a function of flow-rate, described by, for example, the Knox equation [63]:

$$h = A\nu^{1/3} + B\nu + C\nu \quad (27)$$

As peptides have low diffusion rates, the mass transport term, the C term, in the Knox equation will be large and the highest plate number will be obtained at an unusually low flow-rate [1,4] (the choice of flow-rate will be discussed later).

Despite the differences in plate number, band widths are usually comparable for solutes eluted with the same gradient slope, because band width is related both to the plate number and the parameter m, the latter generally increase with increasing molecular mass. This leads to the impression that the variation in plate number is smaller than it actually is. The prediction of band width relies on the estimate of the plate number, and it is therefore essential to consider as many factors as possible in order to maximize the accuracy of predictions.

Based on extensive approximations, expressions have been derived that relate the parameters B and C in the Knox equation to molecular mass and the structural state of the peptide (native/denatured), particle and pore size of the packing material and viscosity of the mobile phase [64]. This approach to the estimation of plate number has the advantages that the variation of plate number with flow-rate can be predicted and that non-ideal band broadening can be detected. Further, this model can aid in the design and evaluation of column materials. However, this model has limited applicability for optimization of separation involving samples of unknown composition as the solute characteristics and Knox parameter A have to be known.

The plate number can instead be determined experimentally from the same gradient runs that are used for estimating a and m. In comparison with the conventional isocratic method for plate number estimation, this method is superior as the determination is made for the organic modifier that its actually used and individual plate numbers can be assigned to the solutes that are separated. In addition, no information is required about the solute or column characteristics and some degree of non-ideal band broadening can be included, making this approach more useful for optimization than the mechanistic model discussed above. However, variations in flow-rate and k' are not accounted for by this approach. It must also be noted that this method

requires that the band width can be measured for all solutes in the sample (see next section).

It is possible that the overestimate of the number of plates made by the conventional isocratic method can be compensated for by a certain correction factor for band width (see Theory). This is a weak argument for the conventional isocratic method, as the difference between the two methods can vary over a wide range, and a correction factor can both over- and under-compensate for an incorrect estimate of plate number.

For comparison, the plate number was determined in this work both by the conventional isocratic method and individually from calibration gradients. For one peptide the plate number was determined with two organic modifiers for a further comparison.

Retention volume, V_g, and band width, W_b

Accurate measurements of the retention volumes for the peaks in the calibration gradients are important in gradient modelling. To determine plate number from calibration gradients, as presented above, the band widths also have to be measured. Determinations of retention volumes and band widths are the initial and most crucial steps in gradient predictions. This can be a difficult problem [65] as evaluation by commercial integrators is often far from perfect [66,67]. Neither do all integrators measure band widths. The problem is not so severe as long as the peaks do not overlap. It is unusual, however, for all solutes to be well separated in the calibration gradients. The overlap of peaks will affect both the determination of retention time (the peak maxima are not equivalent to the retention times), and band width. Incorrect measurements of retention times are a source of significant errors in gradient predictions [39,57].

The problem can be solved, however, by deconvolution of the peaks. Deconvolution means that the overlapping peaks are separated into individual peaks by mathematical methods. Deconvolution can be done without any assumption of peak shape if a diode-array detector is used and the spectra of the non-resolved solutes are different [68–70]. Diode-array detectors are not available in all laboratories and the very advanced deconvolution software is even more scarce. The alternative is to fit a mathematical model to the peaks. Fitting routines for gaussian models are available in many computer-based evaluation programs or can easily be implemented, which was done in this work. Tailing, non-gaussian peaks are sometimes observed owing to secondary interactions [71,72], although gradient elution promotes symmetrical peaks [6]. If tailing peaks are present, the first action should be to alter the chromatographic conditions to prevent tailing, *e.g.*, by increasing the ionic strength [5,73–76] or by adding amines that can block silanol groups [35,77–79] or by altering the pH [5,61,74]. Unfortunately, the tailing can persist. This situation is difficult to handle as their is no simple model for the peak shape and gradient theory does not include tailing. The "brute force" method is to use a gaussian model giving an overestimated band width. The solute will then be predicted to give wide gaussian peaks instead of tailing peaks. This is an incorrect prediction, but still better than if a plate number based on the conventional isocratic method was used.

Choice of calibration gradients

From a small number of gradient runs the parameters a and m can be estimated and results predicted. If V_m and V_d are known, two calibration gradients are sufficient [2,7,37]. To be able to perform calibration it is crucial that the peaks of each solute in the different calibration gradients are matched, so-called peak tracking. The use of three calibration gradients makes this task easier [10,80,81]. Apart from giving more information in general, the use of three calibration gradients also makes it possible to test whether a peak match hypothesis is correct. This is done by determining the parameters a and m from two gradients, and then predicting the third gradient. An incorrect peak match will show up as a large error in the predicted chromatogram. Peak matching is critical in calibration, as mismatched peaks can lead to gross errors in the predictions.

It has been recommended that the slopes of the calibration gradients used should differ by a factor of three or four in order to obtain good

accuracy in the determination [57]. In peptide separations the selectivity often changes with the gradient slope. This makes it difficult to match peaks as the retention order may be very different in the calibration gradients [10]. In optimization work it is practical to restrict oneself to a narrower span of gradient slopes. The consequence is that extrapolation is often unavoidable in predictions for optimization purposes. The influence of extrapolation on the accuracy of predictions was studied in this work.

The calibration gradients must have different slopes, B, or s/F, and the question then arises of whether one should vary the gradient rate [19,20,39] or the flow-rate [10].

The purpose of using gradient theory for optimization is to save experiments and time. It has been shown that for a given gradient slope (%/ml) the peak capacity is at its maximum at a fairly low flow-rate [13,36,82], corresponding to the maximum plate column. This might lead to the conclusion that one should operate at a low flow-rate. However, for a fixed gradient time the maximum peak capacity is obtained at a high flow-rate, as this will result in a gradient with a smaller slope, *i.e.*, resolving more peaks [4,29,83]. The decrease in the plate number is then counteracted by the decreased slope. In other words, when time is important one should work at a high flow-rate and vary the gradient rate for the calibration gradients. The upper limit of flow-rate is usually set by pressure limitations, and many chromatographers find it inconvenient to work at flow-rates higher than 1.0 ml/min for a column of I.D. 4–5 mm. A constant flow-rate in the calibration gradients also means that one source of variation in the plate number is eliminated. It has further been reported that short gradient times and high flow-rates result in high recoveries [13,84,85]. Predictions based on calibration gradients where the gradient rate is varied lead to more accurate results as opposed to variations in flow-rate [22]. The conclusion is, in contradiction to some earlier statements [2,7,10], that the flow-rate should be kept high and constant and that the slope should be varied by alterations in the gradient rate.

METHODS FOR PARAMETER FITTING

Retention volume: the simple method

If V_m and V_d are known and $2.3V_{mg}mBk'_0$ is large, the parameters a and m can be determined from two calibration gradients [2,37] without having to employ numerical methods (if φ_0 is constant):

$$m = \frac{\log(B_1/B_2)}{V_{g1}B_1 - V_{g2}B_2 + (V_m + V_d)(B_2 - B_1)} \quad (28)$$

$$a = \log\left(10^{mB_i(V_{gi}-V_d-V_m)} + 2.3mB_iV_d\right) + m\varphi_0 - \log(2.3V_mmB_1) \quad (29)$$

Note that $2.3V_{mg}mBk'_0$ does not have to be large for the gradients that are to be predicted; eqn. 6 can then be used. The most effective way of keeping $2.3V_{mg}mBk'_0$ large is to start the calibration gradients at a very low content of organic modifier. It is advisable to verify that the assumption is valid after a and m have been estimated. (Obviously the value of $2.3V_{mg}mBk'_0$ is dependent on the estimation of a and m, but a rough estimate of $2.3V_{mg}mBk'_0$ can be made as long it is larger than about 5).

A third gradient, preferably with an intermediate slope, can be used to verify the peak matching. It is possible to improve a rough estimate of V_d by repeating the determination of a and m with different dwell volumes and look for the best fit of the predicted and actual chromatograms for the third gradient [58]. However, the more general and "automatic" method described below is more practical if one wants to use the third gradient for V_d determination.

Retention volume: the advanced method

A more advanced method for parameter fitting is necessary when $2.3V_{mg}mBk'_0$ is small or if one wants to determine V_d by fitting and/or use all three gradients to determine a and m. This calls for a non-linear fitting method. Several methods are well established and are published with program codes [86]. The fitting is best done in two steps, starting by estimating a, m and V_d for every solute. V_d is then taken as an average of all estimates, as it is not solute dependent, and a

new estimate is made of a and m, now keeping V_d fixed. A non-linear fitting routine can, of course, be used even if $2.3V_{mg}mBk'_0$ is large, making this method more general.

Calculating plate number from calibration gradients

Individual plate number for each solute can easily be determined from eqn. (23) if band widths have been measured. From eqn. 23, N can be expressed as

$$N = \frac{16}{W_b^2} \cdot \left\{ \frac{V_{mi}}{V_m} \cdot [V_{mi}(k'_0 + 1)]^2 + \frac{V_{mg}}{V_m} \cdot [V_{mg}(k'_f + 1)JG]^2 \right\} \quad (30)$$

Note that all necessary parameters on the right-hand side can be calculated from the parameters given in the model of the retention volume.

EXPERIMENTAL

Column

A 10-cm × 4 mm I.D. Sephasil C_{18} column (Pharmacia–LKB Biotechnology, Uppsala, Sweden) was used. The column matrix consisted of 5-μm silica with a pore size of 125 Å.

Instrumentation

A system consisting of a Model 2249 low-pressure mixing gradient pump, a Model 2141 dual-wavelength detector (Pharmacia LKB Biotechnology) and a CMA Model 200 autoinjector (CMA Microdialysis, Stockholm, Sweden) was used. The instrumentation was interfaced with an IBM AT3 personal computer for gradient control and data acquisition.

Software

Evaluation of chromatograms, gradient modeling and prediction were all made with in-house software written in the programming environment ASYST (Asyst Software Technologies, Rochester, NY, USA). A modified Gauss–Newton algorithm was used for fitting gaussian models to the chromatograms. The data fitting was done with a simplex algorithm.

Chemicals

Acetonitrile and 2-propanol were of HPLC gradient grade (Merck). Distilled waster was purified using a Milli-Q system (Millipore, Bedford, MA, USA) fitted with an Organex-Q cartridge. Phosphoric acid and ammonia were of analytical-reagent grade (Merck).

Peptides

The synthetic peptides used were kindly donated by Anders Winter at Pharmacia–LKB Biotechnology and are listed in Table II. All peptides were injected individually.

Gradients

Four gradient slopes, 0.5, 1.0, 2.0 and 4.0%/ml, were used. The buffer consisted of 50 mmol/l phosphoric acid adjusted to pH 2.8 with

TABLE II

PEPTIDES USED IN THIS WORK

No.	M_r	pI	Sequence
1	589	3.1	Met–Val–Asn–Pro–Glu
2	571	3.1	Tyr–Glu–Leu–Phe
3	626	8.4	Pro–Leu–Ile–His–Phe
4	1071	6.5	Thr–Pro–Ile–Pro–Arg–Tyr–Pro–Leu–Asp
5	1858	3.9	His–Thr–Asp–Arg–Glu–His–Thr–Ile–Glu–Thr–Asp–Glu–Met–Glu–Asp
6	1729	9.5	Lys–Tyr–Gly–Asn–Leu–Ser–His–Glu–Lys–Gln–His–Gln–Leu–Phe
7	1689	3.1	Gly–Asn–Gly–Gln–Asp–Val–Met–Ala–Leu–Ala–Thr–Ile–Leu–Ser–Trp–Leu
8	1722	9.6	Gln–Leu–Ser–Leu–Ala–Ile–Phe–His–Ser–Thr–Tyr–Trp–Lys–Ala–Gly

ammonia. Acetonitrile was used as organic modifier for all peptides. Peptide 3 was also eluted with 2-propanol gradients. The acetonitrile gradients started at 2 or 4% and the 2-propanol gradient at 1% of organic solvent.

RESULTS AND DISCUSSION

The retention data and estimates of a and m are given in Table III.

Measures of error

The prediction error for retention volume and difference in retention volume is expressed as a percentage of the gradient volume (not the retention volume!). The gradient volume refers to an imagined gradient going from 0 to 50% organic modifier, a common case in peptide separations, with the relevant slope. This measure is rationalized by the fact that in gradient elution the band width does not increase with increase in retention volume. Consequently, an error of for example 0.1 ml in the prediction of retention volume is of equal concern for solutes with small or large retention volumes. Absolute retention volume is also inappropriate as a measure of error as an error of, for example, 0.1 ml is more serious in a high slope gradient, where peaks are narrow. For band widths the relative error is used. The band width is roughly the same for all peaks and decreases with increasing slope, making a relative measure appropriate. In this paper the error is presented as the median and the 90% percentile. The 90% percentile indicates an upper level of the error.

Determination of dead volume, V_m, and dwell volume, V_d

The dead volume was determined by elution of uracil with two mobile phases containing 50% and 30% of acetonitrile. The amounts injected were 0.03 and 3.0 μg. From the results, given in Table IV, it is clear that the elution volume of uracil is lower with 50% of acetonitrile. This is observed for most dead volume markers [42,47,56]. Thus, the increase in elution volume for uracil on going from 50% to 30% of acetonitrile is probably not due to retention but to an actual change in dead volume. The effect of the amount of uracil on the elution volume is very small, although it is statistically significant ($P = 0.05$).

The determination of the dwell volume by parameter fitting was compared with determination employing a gradient of a UV-absorbing solution. The dwell volume was estimated to be

TABLE III

RETENTION DATA

Gradients with acetonitrile. The model parameters a and m are given with the standard deviation in parentheses based on the combination of calibration described under Results and Discussion.

Peptide	Retention volume (ml)								a	m ($\%^{-1}$)
	Starting concentration 2%				Starting concentration 4%					
	Gradient slope (%/ml)				Gradient slope (%/ml)					
	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0		
1	11.55	8.70	6.69	5.29	7.72	6.56	5.52	4.65	1.98(0.14)	0.211(0.022)
2	37.12	22.46	14.07	9.23	33.20	20.47	13.07	8.75	3.38(0.05)	0.130(0.003)
3	35.72	21.36	13.31	8.78	31.71	19.39	12.33	8.25	3.71(0.08)	0.158(0.004)
4	36.75	22.28	13.98	9.20	32.81	20.30	13.00	8.73	3.34(0.05)	0.130(0.003)
5	38.17	21.92	13.25	8.58	33.97	19.87	12.27	8.06	5.39(0.21)	0.244(0.010)
6	24.56	15.15	9.89	6.90	20.54	13.19	8.90	6.37	3.65(0.14)	0.237(0.010)
7	74.15	40.57	22.89	13.59	70.07	38.58	21.90	13.09	6.79(0.13)	0.159(0.003)
8	46.87	26.23	15.36	9.58	42.64	24.12	14.35	9.09	6.78(0.32)	0.259(0.013)

TABLE IV

DETERMINATION OF DEAD VOLUME

The mobile phase consisted of acetonitrile mixed with 50 mmol/l phosphate buffer (pH 2.8). Each determination of V_m was repeated four times. The pooled standard deviation of V_m was 1.8 μl.

Concentration of acetonitrile (%)	Amount of uracil (μg)	V_m (μl)
30	0.03	808
30	3	809
50	0.03	750
50	3	754

2.10 ml from the gradient of a UV-absorbing solution. To find the dwell volume by fitting retention data to the model, more than two calibration gradients have to be made. The overall aim is to keep the number of experiments small, hence the use of three calibration gradients is of most interest. In this work, four different slopes were used, to allow predictions to be made for a gradient not used for calibration. To obtain a realistic situation a combination of three out of four gradients was first selected. For this combination V_d was first determined together with a and m. As one estimate is obtained for each solute, V_d was taken as the average for all solutes. The determination of a and m was then repeated using this V_d as fixed. This procedure was carried out for all combinations of calibration gradients. The average of all V_d estimates based on fitting was 2.47 ml, with V_m set at 0.809 ml.

To evaluate the effect on accuracy, V_g was predicted for the peaks in the gradient not used for calibration. This was repeated for all possible combinations of calibration gradients. It has been concluded by other workers that an error in the estimation of V_m has very little effect on the accuracy of the predictions of retention [24,25,57] and it can be seen in Table V that the variations in the V_m estimates indeed has only a small influence on the prediction error. The best result is obtained using the V_m determined with a mobile phase containing 30% of acetonitrile. This isocratic mobile phase is closer to the average mobile phase during gradient elution in the relevant range and a better estimate of V_m is therefore to be expected.

TABLE V

ERROR IN PREDICTION OF V_g FOR DIFFERENT ESTIMATES OF V_m AND V_d

Eqn. 6 was used for parameter fitting and prediction. The dwell volume was determined, together with the model parameters a and m, by parameter fitting. All combinations of three calibration gradients and starting concentration were considered. The measure of error is explained in the text.

V_m (μl)	Median error/90% percentile (% of V_G)	
	V_d by fit	V_d from UV-absorbing eluent gradient
808	0.40/0.84	0.82/1.51
809	0.40/0.84	0.82/1.51
750	0.40/0.84	0.91/1.69
754	0.40/0.84	0.90/1.68

It is clear that a V_d estimate based on fitting will improve the accuracy, a result that has also been obtained by others [22,23]. When estimating V_d by parameter fitting, the predictions also become less sensitive to small variations in the V_m estimate, as these variations are taken up by V_d. It should be emphasized that the determination of V_d by parameter fitting is sensitive to errors in retention volumes in the calibration gradients. However, as one estimate of V_d is obtained for each solute, the accuracy can be improved by evaluating as many solutes as possible.

Resolution is a more interesting parameter than absolute retention. The error in prediction of resolution is a function of the relative errors in band widths and difference in retention. Sources of prediction error that affect the retention of all solutes in the same direction will therefore have a minor effect on the predictions of resolution. For example, an error in V_d affects the predicted retentions in roughly the same way and the influence on resolution should be small in comparison with the influence on absolute retention.

To evaluate the effect of V_d on resolution, the differences in retention of four peak pairs were predicted and compared with actual values. The results are summarized in Table VI. One can

TABLE VI

ERROR IN PREDICTION OF DIFFERENCES IN V_g BETWEEN PEAK PAIRS FOR THE TWO METHODS FOR ESTIMATION OF V_d

Eqn. 6 was used for parameter fitting and prediction. The dead volume was set at 809 μl. All combinations of three calibration gradients and two starting concentration were considered, as described in the text. The eight peptides were divided into four pairs, *i.e.*, each combination of gradients resulted in four estimates of retention difference. The measure of error is explained in the text.

Method for determination of dwell volume	Median error/90% percentile (% of V_G)
Parameter fitting	0.31/0.68
UV-absorbing eluent gradient	0.36/1.22

conclude that the method for the determination of the dwell volume does not have a large effect on the accuracy for prediction of resolution, which is in agreement with statements made in earlier work [22,25]. The median error is almost the same for both methods but the 90% percentile is higher for the estimation of V_d by the use of a gradient of a UV-absorbing solution. These large errors corresponds to peak pairs that elute early and gradients where φ_0 is large. Under these conditions, the migration during the dwell volume, which is solute dependent, will be more pronounced.

Limit of the $2.3V_{mg}mBk'_0$ approximation

The simple data fit method is based on the assumption that $2.3V_{mg}mBk'_0$ is "large". To establish the limit for this approximation, a and m were determined, with and without this approximation. Predictions where made with eqn. 6, as the approximation is only meaningful in the parameter fitting. The difference between predictions based on fittings with the different models are treated as the error, and the results are presented in Table VII. Note that $2.3V_{mg}mBk'_0$ differs between different calibration gradients, and that only the smallest value is given in Table VII.

In this study, the error is roughly 0.4% of the gradient volume, which is about the same as reported previously [20,22,24,57] (although the error was then expressed as relative retention). In that perspective, one can tolerate a 2.3

TABLE VII

ERRORS IN PREDICTIONS OF V_g DUE TO THE $2.3V_{mg}MBk'_0 \gg 1$ APPROXIMATION IN THE PARAMETER FITTING

The dwell and dead volumes were set at 809 μl and 2.47 ml, respectively. The model parameters a and m were determined by parameter fitting. All combinations of calibration gradients and starting concentration were considered. The error is taken as the difference between predictions based on calibration with and without the approximation. The measure of error is explained in the text.

Peptide	Starting concentration			
	2%		4%	
	$2.3V_{mg}mBk'_0$	Median error/90% percentile (% of V_G)	$2.3V_{mg}mBk'_0$	Median error/90% percentile (% of V_G)
1	6	0.20/0.60	2	0.62/1.15
2	154	0.01/0.06	86	0.02/0.11
3	342	0.00/0.02	164	0.01/0.05
4	143	0.01/0.06	76	0.03/0.12
5	15 900	0.00/0.00	3 650	0.00/0.00
6	304	0.00/0.02	97	0.01/0.05
7	388 000	0.00/0.00	159 000	0.00/0.00
8	403 000	0.00/0.00	75 300	0.00/0.00

$V_{mg}mBk'_0$ value down to *ca.* 50, when using eqn. 8 for parameter fitting. Note that the error decreases with increasing value of m, the guideline given here is only valid for peptides and other solutes with large values of m.

Interpolation versus extrapolation and number of calibration gradients

When optimizing the gradient, it is likely that one wants to predict the result for gradients that are faster or slower than those used for calibration. The question is how far it is acceptable to extrapolate, and whether there is any difference in the accuracy for extrapolation to faster or slower gradients. Extrapolation is compared with interpolation for two and three calibration gradients in Table VIII.

Inherently, extrapolation gives rise to a larger error than interpolation. This has also been observed experimentally [25,39], but from Table VIII it can be seen that extrapolation to faster gradients yields a smaller error than extrapolation to slower gradients. Three calibration gradients give rise to a minor improvement and are also less sensitive to an error in a single retention volume. The practical consequence is that the extrapolation from the calibration gradients is acceptable if the calibration slopes are less than the slopes of the gradients one expects to predict. Low gradient slopes for calibration also simplify peak matching. However, it must be noted that an increase in the experimental error would have a much greater effect on extrapolation than interpolation.

TABLE VIII

ERROR IN PREDICTION OF V_g FOR INTERPOLATION AND EXTRAPOLATION WITH TWO AND THREE CALIBRATION GRADIENTS

The dead and dwell volumes were set at 809 μl and 2.47 ml, respectively. The model parameters a and m were determined by parameter fitting. Combinations of calibration and prediction gradients with the same starting concentration were considered. Gradients are indicated with slope.

Calibration gradient (%/ml)	Predicted gradient	Median error/90% percentile (% of V_G)
Small extrapolation to slower gradients		
1.0, 2.0	0.5	0.45/0.59
1.0, 4.0	0.5	0.27/0.44
2.0, 4.0	1.0	0.58/0.89
1.0, 2.0, 4.0	0.5	0.32/0.45
Large extrapolation to slower gradients		
2.0, 4.0	0.5	0.97/1.45
Small extrapolation to faster gradients		
1.0, 2.0	4.0	0.29/0.47
0.5, 2.0	4.0	0.24/0.39
0.5, 1.0	2.0	0.34/0.45
0.5, 1.0, 2.0	4.0	0.20/0.35
Large extrapolation to faster gradients		
0.5, 1.0	4.0	0.27/0.49
Interpolation		
0.5, 2.0	1.0	0.22/0.30
0.5, 4.0	1.0	0.18/0.32
0.5, 4.0	2.0	0.24/0.37
1.0, 4.0	2.0	0.22/0.35
0.5, 2.0, 4.0	1.0	0.21/0.27
0.5, 1.0, 4.0	2.0	0.26/0.35

Correction factors for band width

The error in band width prediction is related to the model and the plate number error. One would also expect the influence of extrapolation on the error to be different for different correction models.

Three different expressions for band width were evaluated: (i) no correction factor; (ii) J correction according to eqn. 21; and (iii) J correction according to eqn. 22. The cases of extrapolation and interpolation described above were also evaluated. The results are summarized in Table IX.

It is clear that a correction according to eqn. 22 gives the best accuracy. (Eqn. 21 will also fail if the extrapolation is extended even further than in this work, as the polynomial correction contains terms of the fourth order.) However, as the cause of unexpectedly large band broadening at high gradient slopes is uncertain, this observation might be dependent on the instrumentation as one potential reason for this effect is extra-column band broadening.

Determination of plate number

The plate number was first determined by the conventional isocratic method using naphthalene as a test solute and an eluent of acetonitrile–

TABLE IX

ERROR IN PREDICTION OF W_b FOR DIFFERENT BAND WIDTH MODELS AND EXTRAPOLATION WITH TWO AND THREE CALIBRATION GRADIENTS

The dead and dwell volumes were set at 809 μl and 2.47 ml, respectively. The plate number was determined from gradient runs. The model parameters a and m were determined by parameter fitting. All combinations of calibration and prediction gradients with the same starting concentration were considered.

Calibration gradients	Predicted gradient	Median/90% percentile of relative errors (%)		
		Correction method		
		None	Eqn. 21	Eqn. 22
Small extrapolation to slower gradients				
1.0, 2.0	0.5	29/41	16/27	10/19
1.0, 4.0	0.5	38/48	21/28	12/19
2.0, 4.0	1.0	35/39	17/19	8/11
1.0, 2.0, 4.0	0.5	42/56	22/34	13/22
Large extrapolation to slower gradients				
2.0, 4.0	0.5	53/63	26/36	14/24
Small extrapolation to faster gradients				
1.0, 2.0	4.0	37/40	17/21	7/11
0.5, 2.0	4.0	47/52	22/26	10.15
0.5, 1.0	2.0	33/43	17.24	10.15
0.5, 1.0, 2.0	4.0	47/51	21/26	8/13
Large extrapolation to faster gradients				
0.5, 1.0	4.0	56/62	27/31	12/17
Interpolation				
0.5, 2.0	1.0	2/8	2/7	3/7
0.5, 4.0	1.0	9/16	5/12	3/10
0.5, 4.0	2.0	15/27	8/16	5/11
1.0, 4.0	2.0	5/12	4/9	3/8
0.5, 2.0, 4.0	1.0	24/32	13/18	7/11
0.5, 1.0, 4.0	2.0	24/32	12/18	7/11
Overall		32/52	15/27	8/16

water (60:40, v/v), $k' = 4.5$, resulting in a plate number of 8610.

The use of calibration gradients for determinations of plate number (eqn. 30) allows individual plate numbers to be estimated for the organic modifier actually used. Every calibration gradient results in estimates of the individual plate numbers. A median of $\sqrt{N}$ from the calibration is calculated for each solute. $\sqrt{N}$ is chosen for the median, instead of N, because $\sqrt{N}$ is inversely proportional to the estimate of W_b, from which a large part of the error arises. The median is selected instead of the average as there is a risk of gross error in the band width at extremely low resolutions or signal-to-noise ratios. The results are given in Table X. The disparity between the isocratic and gradient method is large and will be even greater for larger peptides.

The effect on the prediction of W_b from plate number estimates by the conventional method and by parameter fitting is shown in Table XI. As expected, the accuracy of the predictions is greatly improved by using plate numbers determined from the calibration gradients. The gain will be larger for larger peptides.

TABLE X

PLATE NUMBERS FOR NINE PEPTIDES DETERMINED BY PARAMETER FITTING FROM FOUR GRADIENTS FOR TWO DIFFERENT ORGANIC MODIFIERS

Band width correction was made according to eqn. 22. The dead and dwell volumes were set at 809 μl and 2.47 ml, respectively. The model parameters a and m were determined by parameter fitting. Only the lower starting concentration for each organic modifier was considered. The plate number estimated by the conventional isocratic methods was 8610.

Peptide	Plate number	
	Acetonitrile	2-Propanol
1	3230	
2	5780	3520
3	4500	
4	5690	
5	2100	
6	1640	
7	3100	
8	1880	

TABLE XI

ERROR IN PREDICTION OF W_b BASED ON DIFFERENT ESTIMATES OF PLATE NUMBER

Band width correction was made according to eqn. 22. The dead and dwell volume were set at 809 μl and 2.47 ml, respectively. The model parameters a and m were determined by parameter fitting. All combinations of three calibration gradients and one prediction gradient with the same starting concentration were considered.

Method for determination of plate number	Median error/90% percentile (%)
Conventional, isocratic	43/72
Parameter fitting, individual	8/17

The error in the prediction of band width is related to the choice of correction factor. This evaluation of the method for determination of plate number was done with correction according to eqn. 22.

In the case of overlapping peaks, the band widths and retention volumes have to be determined after a deconvolution, as described under Theory. Deconvolution is not an easy task, and low signal-to-noise ratios, extremely low resolutions or large area ratios can make the task almost impossible. In addition, the necessary software may not be available to all chromatographers. For non-deconvoluted peaks, the plate number can be taken as the average of the plate number for the solutes in the sample that are well separated. The inaccuracy in retention prediction can be reduced by using more than two calibration gradients.

Test peptides

The purpose of the eight peptides used in this work was to represent possible variations that one can find in peptides. These peptides are still limited: none contains cysteine or methionine and they are not longer than sixteen amino acids. The consequence is that they do not exhibit any higher structure and have fairly high diffusion coefficients. They are chemically well behaved, with no denaturation, rearrangement or oxidation.

It is known that some peptides are not well behaved; typically they undergo permanent

TABLE XII

ERROR SOURCES

Source	Comment
Incorrect gradient formation	Should be checked by running a UV-absorbing gradient without column. Generally small with modern equipment [57,58]
Gradient rounding due to mixing volume	The mixer should have a volume that is less than 20% of the total volume of the gradient. This is rarely a problem with ordinary instrumentation, but can be serious with micro columns. In addition it can pose a difficulty when multi-segmented gradients are used [58]
Incomplete column equilibration	It has been found that 15 column volumes of initial solvent are needed to wash the column completely between the gradients [24]. Incomplete column equilibration will only affect early-eluting solutes
Solvent demixing	Small [20,24,57], and can be further minimized by not starting the gradient with pure buffer, *i.e.*, by having $\varphi_0 > 0\%$

structural changes, their retention parameters change or they undergo slow inverconversions between two or more conformations [3,7,87–90]. This leads to excess band broadening or multiple peaks and can cause deviations between predicted and actual values. These cases can, however, usually be recognized in the calibration gradients. Very few cases of large deviations have been reported [7].

Other sources of error

There are general sources of error that are not related to the choice of the chromatographic models and methods of determining the parameters. They are listed in Table XII together with a judgement of their importance. It was not the aim of this work to explore these issues, and papers have already been published that treat this topic [20,24,25,31,37,57,58].

In the reversed-phase liquid chromatography of peptides, mobile phases containing trifluoroacetic acid (TFA) are popular. It has been shown that these mobile phases degrade the column [91]. If TFA is used as a mobile phase additive it is therefore important that the time between the calibration and prediction gradient is small [20,57].

CONCLUSIONS

It has been confirmed that highly accurate predictions of retention volumes, band widths and resolutions can be made from a small number of calibration gradients if the right precautions are taken. There are various versions of the expressions for retention volume and band width in the literature, most of them being different approximations based on the same fundamental theory. From a practical point of view, considering the tools for calculation that are available today, there is only one approximation that is meaningful. By choosing the right conditions for the calibration gradients, *i.e.*, low starting concentration of organic modifier, one can ensure that this approximation is valid. The determination of the model parameters then becomes simple.

Determination of the dead volume as the elution volume for uracil is simple and was found to be adequate. The dwell volume can be estimated from a gradient run, without a column, with UV-absorbing eluents. The alternative approach is to estimate the dwell volume from parameter fittings of retention data based on three or more calibration gradients, if non-linear fitting routines are available. In agreement with earlier work, it has been shown that determination of the dwell volume by fitting results in more accurate predictions of absolute retention times. The improvement in the accuracy of prediction of differences in retention between peak pairs is, however, minor.

The accuracy of band width prediction can be greatly improved by assigning an individual plate

number to each solute. This is especially important in peptide work, as the plate number is generally much smaller than for small organic compounds, and also varies between peptides. Plate numbers can either be derived from approximating mechanistic models or be determined experimentally. The latter approach is more appropriate for optimization purposes, as it requires no knowledge about the solute or column and can tolerate some non-ideal band broadening. Deviations from the fundamental band width model have been observed by other workers. This was confirmed in this work and the different empirical correction methods suggested in the literature have been evaluated. The simplest approach gave the highest accuracy in this study.

The initial step in making predictions is to evaluate the calibration gradients. A correct match of peaks between runs is crucial, and this task is much simplified if three calibration gradients are run. Determinations of retention volumes and band widths (the latter is needed if individual plate numbers are to be estimated) is difficult in the case of overlapping peaks. Here a simple deconvolution based on a gaussian model is suggested. It is recommended that the calibration gradients are run at a constant and high flow-rate. This will not correspond to the maximum plate number but to the highest peak capacity per unit time. Predictions based on calibration gradients with constant flow-rate are also more accurate.

Gradient prediction is mainly a tool for optimization of the gradient slope. Extrapolation is then often inevitable. It is shown here that extrapolations to faster gradients are associated with smaller errors than extrapolations to slower gradients, suggesting that slow calibration gradients should be preferred.

In summary, gradient prediction is accurate and useful in peptide chromatography and, following the guide-lines presented in this work, its implementation is made simpler.

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SYMBOLS

φ	Concentration of organic modifier (%)
φ_0	Starting concentration of organic modifier (%)
ν	Reduced velocity
a	Model parameter
B	Gradient slope (%/ml)
b	Gradient steepness
F	Flow-rate (ml/min)
G	Peak compression factor
k'_0	k' at the starting concentration of organic modifier
k'_f	k' when the solute leaves the column
m	Model parameter ($\%^{-1}$)
N	Plate number
s	Gradient rate (%/min)
t	Time (min)
V	Pumped volume (ml)
V_g	Retention volume, gradient elution (ml)
V_G	Gradient volume (ml)
V_d	Dwell volume (ml)
V_m	Dead volume (ml)
W_b	Peak width at base (ml)

REFERENCES

1 L.R. Snyder, M.A. Stadalius and M.A. Quarry, *Anal. Chem.*, 55 (1983) 1412A.
2 M.A. Stadelius, H.S. Gold and L.R. Snyder, *J. Chromatogr.*, 296 (1984) 31.
3 M.-I. Aguilar, A.N. Hodder and M.T.W. Hearn, *J. Chromatogr.*, 327 (1985) 115.
4 J.L. Meek and Z.L. Rossetti, *J. Chromatogr.*, 211 (1981) 15.
5 M.J. O'Hare and E.C. Nice, *J. Chromatogr.*, 171 (1979) 209.
6 L.R. Snyder, in Cs. Horváth (Editor), *High Performance Liquid Chromatography: Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 207.
7 L.R. Snyder and M.A. Stadalius, in Cs. Horváth (Editor), *High Performance Liquid Chromatography: Advances and Perspectives*, Vol. 4, Academic Press, New York, 1986, p. 195.
8 P. Jandera and J. Churáček, *Gradient Elution in Column Liquid Chromatography, Theory and Practice*, Elsevier, Amsterdam, 1985.

9 B.F.D. Ghrist and L.R.Snyder, *J. Chromatogr.*, 459 (1988) 25.
10 J.L. Glajch, M.A. Quarry, J.F. Vasta and L.R. Snyder, *Anal. Chem.*, 58 (1986) 280.
11 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 207.
12 P.J. Schoenmakers, H.A.H. Billiet, R. Tijssen and L. de Galan, *J. Chromatogr.*, 149 (1978) 519.
13 M.T.W. Hearn and B. Grego, *J. Chromatogr.*, 255 (1983) 125.
14 M.T.W. Hearn and B. Grego, *J. Chromatogr.*, 266 (1983) 75.
15 P.J. Schoenmakers, H.A.H. Billiet and L. de Galan, *J. Chromatogr.*, 185 (1979) 179.
16 J.W. Dolan, J.R. Gant and L.R. Snyder, *J. Chromatogr.*, 165 (1979) 31.
17 P. Jandera and J. Churáček, *J. Chromatogr.*, 192 (1980) 1.
18 R.A. Harwick, C.M. Grill and P.R. Brown, *Anal. Chem.*, 51 (1979) 34.
19 B.F.D. Ghrist and L.R. Snyder, *J. Chromatogr.*, 459 (1988) 43.
20 J.W. Dolan, D.C. Lommen and L.R. Snyder, *J. Chromatogr.*, 485 (1989) 91.
21 J. Schmidt, *J. Chromatogr.*, 485 (1989) 421.
22 J.D. Stuart, D.D. Lisi and L.R. Snyder, *J. Chromatogr.*, 485 (1989) 657.
23 S. Heinisch, J.-L. Rocca and M. Kolosky, *Chromatographia*, 29 (1990) 482.
24 M.A. Quarry, R.L. Grob and L.R. Snyder, *J. Chromatogr.*, 285 (1984) 19.
25 L.R. Snyder and M.A. Quarry, *J. Liq. Chromatogr.*, 10 (1987) 1789.
26 S.A. Tomellini, R.A. Harwick and H.B. Woodruff, *Anal. Chem.*, 57 (1985) 811.
27 Y. Sakamoto, N. Kawakami and T. Sasagawa, *J. Chromatogr.*, 442 (1988) 69.
28 M. Kunitani, D. Johnson and L.R. Snyder, *J. Chromatogr.*, 371 (1986) 313.
29 M.T.W. Hearn and M.I. Aguilar, *J. Chromatogr.*, 359 (1986) 31.
30 L.R. Snyder, M.A. Quarry and J.L. Glajch, *Chromatographia*, 24 (1987) 33.
31 M.A. Quarry, R.L. Grob and L.R. Snyder, *J. Chromatogr.*, 285 (1985) 1.
32 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 223.
33 L.R. Snyder and D.L. Saunders, *J. Chromatogr. Sci.*, 7 (1969) 195.
34 H. Poppe and J. Panakker, *J. Chromatogr.*, 204 (1981) 77.
35 M.A. Stadelius, H.S. Gold and L.R. Snyder, *J. Chromatogr.*, 327 (1985) 27.
36 M.A. Stadelius, M.A. Quarry and L.R. Snyder, *J. Chromatogr.*, 327 (1985) 93.
37 M.A. Quarry, R.L. Grob and L.R. Snyder, *Anal. Chem.*, 58 (1986) 907.
38 M.T.W. Hearn and M.I. Aguilar, *J. Chromatogr.*, 352 (1986) 35.
39 J.W. Dolan, L.R. Snyder and M.A. Quarry, *Chromatographia*, 24 (1987) 261.
40 A. Alhedai, D.E. Martire and R.P.W. Scott, *Analyst*, 114 (1989) 869.
41 J.H. Knox and R. Kaliszan, *J. Chromatogr.*, 349 (1985) 211.
42 H. Engelhardt, H. Müller and B. Dreyer, *Chromatographia*, 19 (1984) 240.
43 A. Malik and K. Jinno, *Chromatographia*, 30 (1990) 135.
44 R.J. Smith, C.S. Nieass and M.S. Wainwright, *J. Liq. Chromatogr.*, 9 (1986) 1387.
45 P.C. Sadek, P.W. Carr and L.D. Bowers, *LC Mag.*, 3 (1985) 590.
46 B.A. Bidlingmeyer, F.V. Warren, A. Weston and C. Nugent, *J. Chromatogr. Sci.*, 29 (1991) 275.
47 A.M. Krstulovic, H. Colin and G. Guiochon, *Anal. Chem.*, 54 (1982) 2438.
48 H. Wätzig and S. Ebel, *Chromatographia*, 31 (1991) 544.
49 R.J. Laub and S.J. Madden, *J. Liq. Chromatogr.*, 8 (1985) 173.
50 J.P. Larmann, J.J. Destefano, A.P. Goldberg, R.W. Stout, L.R. Snyder and M.A. Stadalius, *J. Chromatogr.*, 255 (1983) 163.
51 N. Tanaka, K. Kimata, Y. Mikawa, K. Hosoya, T. Araki, Y. Ohtsu, Y. Shiojima, R. Tsuboi and H. Tsuchiya, *J. Chromatogr.*, 535 (1990) 13.
52 R.V. Lewis, A. Fallon, S. Stein, K.D. Gibson and S. Udenfriend, *Anal. Biochem.*, 104 (1980) 153.
53 R.R. Walters, *J. Chromatogr.*, 249 (1982) 19.
54 R.V. Lewis and D. Dewald, *J. Liq. Chromatogr.*, 5 (1982) 1367.
55 J.D. Pearson, W.C. Mahoney, M.A. Hermodson and F.E. Regnier, *J. Chromatogr.*, 207 (1981) 325.
56 R.M. McCormick and B.L. Karger, *Anal. Chem.*, 52 (1980) 2249.
57 B.F.D. Ghrist, B.S. Cooperman and L.R. Snyder, *J. Chromatogr.*, 459 (1988) 1.
58 L.R. Snyder and J.W. Dolan, *LC·GC Int.*, 3(10) (1990) 28.
59 K.D. Nugent, W.G. Burton, T.K. Slattery, B.F. Johnson and L.R. Snyder, *J. Chromatogr.*, 443 (1988) 381.
60 A.K. Taneja, S.Y.M. Lau and R.S. Hodges, *J. Chromatogr.*, 317 (1984) 1.
61 C.T. Mant and R.S. Hodges, *J. Liq. Chromatogr.*, 12 (1989) 139.
62 R.W. Stout, J.J. DeStefano and L.R. Snyder, *J. Chromatogr.*, 282 (1983) 263.
63 J.H. Knox, *J. Chromatogr., Sci.*, 15 (1977) 353.
64 M.A. Stadalius, B.F.D. Ghrist and L.R. Snyder, *J. Chromatogr.*, 387 (1987) 21.
65 A.N. Papas, *CRC Crit. Rev. Anal. Chem.*, 20 (1989) 359.
66 A.N. Papas and M.F. Delaney, *Anal. Chem.*, 59 (1987) 54A.
67 A.N. Papa and T.P. Tougas, *Anal. Chem.*, 62 (1990) 234.
68 B.G.M. Vandeginste, G. Kateman, J.K. Strasters, H.A.H. Billiet and L. de Galan, *Chromatographia*, 24 (1987) 127.
69 G.G.R. Seaton and A.F. Fell, *Chromatographia*, 24 (1987) 208.

70 M. Maeder, *Anal. Chem.*, 59 (1987) 527.
71 C.T. Wehr, R.P. Lundgard and K.D. Nugent, *LC·GC Int.*, 2(3) (1989) 24.
72 W.G. Burton, K.D. Nugent, T.K. Slattery, B.R. Summers and L.R. Snyder, *J. Chromatogr.*, 443 (1988) 363.
73 H. Engelhardt, G. Appelt and E. Schweinheim, *J. Chromatogr.*, 499 (1990) 165.
74 C.T. Mant and R.S. Hodges, *Chromatographia*, 24 (1987) 805.
75 W.S. Hancock and J.T. Sparrow, *J. Chromatogr.*, 206 (1981) 71.
76 H. Engelhardt and D. Mathes, *Chromatographia*, 14 (1981) 325.
77 C.D. Leach, M.A. Stadalius, J. Berus and L.R. Snyder, *LC·GC Int.*, 1(5) (1988) 22.
78 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299.
79 K.A. Cohen, J. Chazaud and G. Calley, *J. Chromatogr.*, 282 (1983) 423.
80 J.W. Dolan, *LC·GC Int.*, 3(7) (1990) 17.
81 I. Molnar, R. Boysen and P. Jekow, *J. Chromatogr.*, 485 (1989) 569.
82 G. Jilge, R. Janzen, H. Giesche, K.K. Unger, J.N. Kinkel and M.T.W. Hearn, *J. Chromatogr.*, 397 (1987) 71.
83 N.H.C. Cooke, B.G. Archer, M.J. O'Hare, E.C. Nice and M. Capp, *J. Chromatogr.*, 255 (1983) 115.
84 M.J. O'Hare, M.W. Capp, E.C. Nice, N.H.C. Cooke and B.G. Archer, *Anal. Biochem.*, 126 (1982) 17.
85 P.C. Sadek, P.W. Carr, L.D. Bowers and L.C. Haddad, *Anal. Biochem.*, 153 (1986) 359.
86 W.H. Press, B.P. Flannery, S.A. Teukolsky, W.T. Vetterling, *Numerical Recipies in C*, Cambridge University Press, Cambridge, 1988.
87 L.R. Snyder, in K.M. Gooding and F.E. Regnier (Editors), *HPLC of Biological Macromolecules: Methods and Applications*, Marcel Dekker, New York, 1990, p. 231.
88 S.A. Cohen, K.P. Benedek, S. Dong, Y. Tapuhi and B.L. Karger, *Anal. Chem.*, 56 (1984) 217.
89 M.T.W. Hearn and B. Grego, *J. Chromatogr.*, 296 (1984) 61.
90 M.A. Stadalius, M.A. Quarry, T.H. Mourey and L.R. Snyder, *J. Chromatogr.*, 358 (1986) 17.
91 J.L. Glajch, Kirkland and J. Köhler, *J. Chromatogr.*, 384 (1987) 81.