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Optimization strategy for reversed-phase liquid chromatography of peptides

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ABSTRACT

An optimization strategy for the separation of peptides from a complex matrix by reversed-phase liquid chromatography is presented and illustrated with an example. The aim is to find the mobile phase system, *i.e.* buffer and organic modifier used for gradient formation, that admits the steepest gradient with sufficient resolution. The result is a rapid separation where the detection limits are low and the loading capacity is high. This strategy is a hybrid between experimental design used for optimizing the selection of the mobile phase system and gradient theory used for gradient predictions.

INTRODUCTION

Reversed-phase liquid chromatography, which was introduced in the mid-1970s, soon became the most popular technique in liquid chromatography. The first attempts to use this separation mode for peptides were not very successful, as low efficiencies and bad peak shapes were observed [1,2]. These initial problems where later solved by improved column technology and reversed-phase liquid chromatography is now a standard separation technique for peptides and small proteins.

Reversed-phase separations of peptides and proteins are still not trivial, however, and the life sciences continue to present extremely demanding applications for chromatography. Problems such as denaturation [3–5], low recoveries [4,6–9], ghost peaks [6,8], low column stability [10,11] and highly complex samples [12,13] are common problems for the bio-chromatographer. Consequently, method development tends to be highly elaborate.

To aid in method development, several optimization strategies for liquid chromatography have been presented; for an overview, see refs. 14–17. These strategies generally fall into two categories, as follows.

Methods based on retention models. In this category of strategies, the retention of all solutes in the sample is modelled. This is attractive as chromatograms can be simulated and the methods do not require that chromatograms are directly graded by some response function (see below). The models that are made can be either empirical, simple polynomial, or have theoretical foundations. The optimum is located either according to some criteria, applied after the modelling, or the user chooses conditions after visual inspection of simulated chromatograms. The disadvantage is that these strategies demand that the variations in retention times for the components in the sample can be followed as the separation conditions are altered. This so-called peak tracking, illustrated in Fig. 1, can be anything from trivial to impossible [18–25]. A number of factors determine the extent to which

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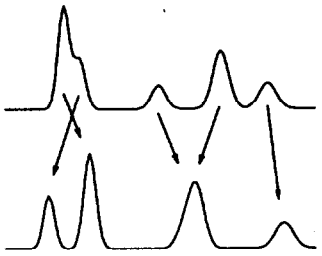

Fig. 1. Peak tracking. The peaks in chromatograms obtained under different conditions are matched.

peak tracking can be applied *i.e.*, the complexity of the sample, which variables are altered and what detection method is selected.

Methods not based on retention models. These methods are straightforward to use, but usually require more experiments than the previous category. No peak tracking is needed and any quantitative variable can be optimized. A response can be seen as a grade or a quality measure for specific separation conditions. This measure is determined from the chromatogram by some response function, *e.g.*, the sum of all resolutions. This response can then be optimized with a search method such as the simplex method [26,27] or by making an empirical model of the response, a so-called response surface. Unfortunately, both methods have significant drawbacks. The simplex method will find an optimum, but it might only be a local optimum. An empirical model of the response can be extremely difficult to make from response values alone, as the response surfaces are often unsmooth [28–31]. A third possibility is to determine the response at various conditions according to an experimental design and then simply take the conditions that result in the highest response as the optimum, without making any model [32]. This last strategy is sometimes referred to as grid searching. It must also be emphasized that it can be difficult to formulate an adequate response function or optimization criteria [33,34]. This will be more of a problem in this category of optimization methods as they rely heavily on a response function.

The large number of optimization strategies proposed in the literature may seem confusing but they are complementary as they aim for different situations. The chromatographer must identify the separation problem and choose optimization methods accordingly. A key question is whether peak tracking is possible or not. The most powerful instrumental tool for peak tracking is multi-channel detection, usually diode-array detection, preferably combined with software that mathematically can resolve peaks [35–37], so-called deconvolution. The outcome is a pure spectrum for every peak, which can support peak tracking. Optimization strategies based on peak tracking with diode-array detection have therefore received considerable attention [38–41]. Unfortunately these strategies are only guaranteed to work if the solutes have different spectra and some degree of resolution. It is also known that a UV spectrum is affected by pH and solvent.

The optimization strategy presented in this work mainly aims at a situation where a small number of peptides are to be separated from an unknown, complex matrix. Typically this means analytical or preparative separation of peptides in biological material such as body fluids, tissue, food or beverages. The mobile phase system, *i.e.*, buffer and organic modifier, and the gradient slope are optimized. The aim is to find separation conditions that allow the separation to be made with a fast gradient. A fast gradient means fast separation, low detection limits and high load capacity.

Peak tracking is difficult in these applications, owing to the complexity of the sample and the fact that spectra of peptides are often identical in the short-wavelength UV range and the differences in the 240–280-nm range are difficult to detect as the absorbance is low. Diode-array detectors are thus of limited use. In addition, they are generally less sensitive than the conventional UV detectors and the deconvolution software is not yet widely available.

The optimization strategies that have been developed so far have either been made for simple matrices with small molecules separated with isocratic elution or have been dedicated to gradient optimization. In this paper, a strategy for peptide separation that optimizes both the mobile phase system and the gradient slope is proposed. Peak tracking is applied to a limited

extent and complex matrices can therefore be handled.

In the proposed optimization strategy, the retention is modelled as a function of gradient slope and response optimization is used for the mobile phase systems. Retention models for gradient elution that are based on chromatographic theory have been found to be highly accurate [42–47]. The input data that are needed can easily be obtained from two or three experiments. On the other hand, variables such as pH, concentration of ion-pairing reagent and composition of organic modifier affect the chromatography in such a way that modelling and peak tracking become more difficult. In addition, there are also experimental limitations on how much these variables can be altered. A strategy based on experimental design and the use of only response values is necessary when these mobile phase variables are used in complex peptide separations. In this work, a mobile phase system that allows variations of pH, concentration of ion-pairing reagent and composition of organic modifier is used.

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 particles with a pore size of 125 Å. The dead volume was determined to be 809 μl by injection of 3 μg of uracil. The column was operated at a flow-rate of 1 ml/min in all experiments.

Mobile phases

All buffers consisted of 50 mmol/l phosphoric acid. The pH was adjusted with ammonia. When trifluoroacetic acid (TFA) was used, it was added before the pH adjustment. The TFA concentration refers to the total volume, including the organic solvent. Consequently, the amount of TFA added to the buffer to be mixed with organic solvent (eluent B or C) was larger than the amount added to eluent A (pure buffer). Gradients were made by mixing three eluents, A, B and C, using the low-pressure mixing facility of the gradient pump. Eluent A was neat buffer and solutions B and C consisted of 50 vol.% of organic solvent in the buffer. The organic solvent was pure acetonitrile for eluent C and a 50:50 mixture of acetonitrile and 2-propanol for eluent B.

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 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, determination of the parameters in the gradient model and predictions were all made with in-house written software using the programming environment ASYST (Asyst Software Technologies, Rochester, NY, USA) running on an IBM PS/2 Model 55SX computer. A modified Gauss–Newton algorithm was used for the deconvolution of overlapping peaks.

Chemicals

Acetonitrile and 2-propanol were of HPLC gradient grade (Merck). Distilled water 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). Trifluoroacetic acid was of spectroscopic grade (Uvasol; Merck) and was distilled before use.

Sample preparation

A 50-mg amount of myoglobin from horse heart (M-1882; Sigma, St. Louis, MO, USA) was dissolved in 5 ml of a 0.1 mol/l solution of ammonium hydrogencarbonate. A 50-mg amount of Trypsin (TPCK treated; Sigma, St. Louis, MO, USA) was dissolved in 2.5 ml of 0.1 mol/l hydrochloric acid and 125 μl of this trypsin solution was added to the myoglobin solution. Digestion was carried out at 37°C for 3 h and was stopped by addition of 1 ml of 30% (v/v) acetic

acid. The digest was spiked with a solution of angiotensin II resulting in a final concentration of 0.14 mg/ml.

RESULT AND DISCUSSION

Gradient predictions

Using gradient theory, it is possible to predict accurately, from two or more gradients, the retention volume and band width at various gradient slopes. The number of experiments for optimization can then be greatly reduced. In this work, gradient prediction was used to determine an adequate gradient steepness for a given mobile phase system, as will be described in the next section.

The fundamental theory of gradient elution in reversed-phase liquid chromatography is well established; a complete presentation of the methodology can be found in publications by Snyder and Stadalius [48,49] and Jandera and Churáček [50]. Most implementations of gradient theory, including this work, rely on a linear relationship between the logarithm of the capacity factor of (k') and the percentage of organic modifier, φ:

$$\log k' = a - m\varphi$$

The band width is assumed to be related to k'_f, the instantaneous value of k' as the solute leaves the column [48,51];

$$W_{bg} = 1.1 \frac{4V_m}{\sqrt{N}} \cdot (k'_f + 1)$$

where k'_f is given by

$$k'_f = 1/[2.30b + 1/(k'_0)]$$

k'_0 is k' in the starting eluent, given by

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

The parameter b (gradient steepness) is a function of both the gradient slope and the solute-dependent parameter m, and can be regarded as the apparent slope or the acceleration of a solute as it is exposed to the gradient. This measure is conceptually more difficult (see the discussion by Lundell [47]) than the slope (%/ml) or rate (%/min), but both k'_f and band width are more closely related to this parameter than the ordinary measures. The gradient steepness b is related to the slope, B, and rate, s, as

$$b = V_m mB = V_m m(s/F)$$

The options in the implementation and application of gradient theory have already been presented and evaluated for the case of peptides [47]. The recommendations made were followed here, including the use of individual plate numbers for each solute. Band widths for these calculations were calculated by fitting gaussian functions to the peaks. This method allows band widths to be determined even if the peaks are overlapping.

Aim of optimization

The choice of a quality measure, a so-called response function, is crucial in optimization. There is no universal response function as there are different demands on different separations. The strategy presented in this work is for an optimization of the mobile phase system and the gradient slope, where the gradient is linear and non-segmented. These simple gradients are adequate for the separation of a few peptides from a complex sample. In this work, one peptide is considered but extension to several peptides can be discussed in a similar fashion.

With the proposed optimization strategy, a minimum resolution is chosen. The resolution for the peptide of interest is calculated as

$$R_s = 2 \cdot \frac{V_{g,2} - V_{g,1}}{W_{b,2} + W_{b,1}}$$

and refers to its neighbouring peaks.

For a specific mobile phase system, it is calculated, using gradient theory, how fast a gradient can be run while still obtaining the desired resolution. This limiting gradient steepness is the response. The aim of the optimization is to find the mobile phase system that allows the fastest gradient. A fast gradient means fast separation, symmetrical peaks, low detection limit and high loading capacity, and thus the choice of response. In addition, it has been observed that the recovery increases with decreasing gradient time [52–54].

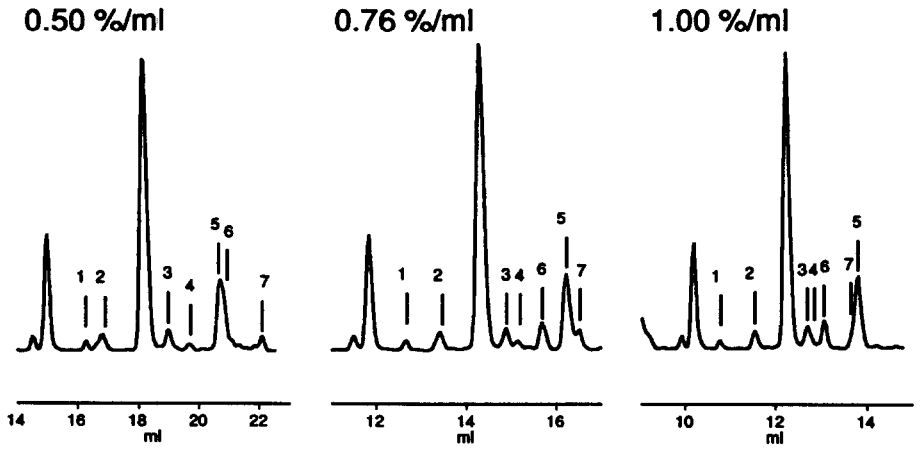

Fig. 2. Chromatogram obtained at 215 nm of an angiotensin II-spiked sample with various gradient slopes. Angiotensin II is the largest peak. The peaks are labelled with numbers. 50 mmol/l phosphate (pH 2.8) was used as buffer and acetonitrile as organic modifier.

The following example illustrates the method. A sample containing angiotensin II as the peptide of interest was eluted using three gradients with different slopes. The angiotensin II peak is shown in Fig. 2 with several interferences at similar retention volumes. Peaks are then matched and the parameters a, m and plate number are determined by data fitting. The resolutions between angiotensin II and the potential interferences are then calculated as a function of gradient slope (Fig. 3). The maximum gradient steepness, b, that gives the desired resolution can then be determined. For example, with a desired resolution of 1.5 the actual maximum gradient slope would be 1.8 ml/min, corresponding to a steepness of 0.26. This steepness is the response for this particular mobile phase system. The choice of response is a central part of this strategy and it is important to understand that the responses that are presented are predicted gradient steepnesses that give the desired resolution, and not gradients that actually have been used.

The size of the smallest interfering peaks that should be taken into account, the limit of concern, is an important issue. In this work, this limit of concern was set to 2% of the analyte peak area at 215 nm. The reasons for this limit are that smaller peaks are difficult to detect and to track in complex samples. It is possible, of course, to optimize the gradient slope by a method not based on gradient predictions, making tracking unnecessary. Unfortunately, it would then be extremely difficult to discover co-elution of very small peaks, making this kind of strategy both time consuming and unreliable.

Several alternative measures of resolution, apart from the traditional one used in this work, have been proposed. The alternative measures,

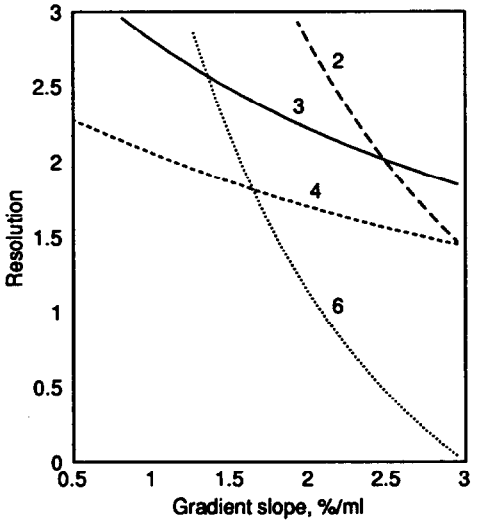

Fig. 3. Resolution between angiotensin II and interferents, numbered according to Fig. 2, as a function of gradient slope, B. Note that gradient steepness, b, is given by $V_m mB$. The parameter m was estimated to be 0.190 for angiotensin II. The resolution of angiotensin II and interferents 1, 5 and 7 is >3.0 in the presented range of gradient slope.

e.g., peak-to-valley ratio, P_v, account for overlap which is dependent on relative areas [33]. R_s was chosen as the measure in this work as it does not vary with the relative amount of the matrix and it is easy to calculate from the gradient predictions.

Selection of mobile phase systems for peptide chromatography

In peptide chromatography, the demands on the mobile phase are high. The mobile phase should allow detection in the 210–230-nm range where the peptide bond absorbs and be highly pure as gradient elution is sensitive towards impurities. It is also essential that the mobile phase minimizes secondary interactions, which lead to bad peak shapes, low efficiency, adsorption, memory effects, low recoveries and bad reproducibility [55–59]. In addition, it is desirable that the mobile phase is not aggressive towards the column to prevent leakage of stationary phase and a shortened column lifetime. Finally, in preparative applications it is convenient if the mobile phase is easy to desalt.

The first attempts at peptide separations by reversed-phase liquid chromatography were not successful owing to a lack of ionic strength in the mobile phase. Addition of acid improved the situation as the ionic strength increased and the pH was lowered, which also reduced silanol interactions [60,61]. Phosphoric acid soon became a popular additive and in 1978 Rivier [62] introduced phosphoric acid with the pH adjusted with triethylamine as buffer. By adding triethylamine, the silanol groups were effectively blocked, which further reduced the silanol interaction.

In 1980, Bennett *et al.* [63] described the use of trifluoroacetic acid (TFA) as a mobile phase additive. TFA is volatile and can be desalted by freeze-drying, therefore making it extremely convenient for preparative separations. TFA also works as an ion-pairing reagent, giving increased retention [64,65]. TFA soon became the standard additive for peptide separations. In the late 1980s several papers appeared that reported some disadvantages with TFA, such as low column stability [10,11,66], significant stationary phase leakage from the column [10], bad peak shapes [6,56,67,68] and low recoveries [6,69]. More recently, formic acid has been suggested as an alternative additive despite its relatively high UV absorbance [70].

The choice of mobile phase is also related to column properties. The interaction with silanol groups, which has been the cause of many problems, is commonly reduced by end-capping and deactivation of the packing material [71–73]. Improvements have also been made in the stability of these silica-based reversed-phase columns [74,75] and, in addition, polymer-based reversed-phase columns with good chromatographic performance are being introduced [11,76–78]. Despite column advances, problems still exist and it is clear that there is no general and perfect mobile phase for the reversed-phase chromatography of peptides.

In this work, phosphoric acid was used as a buffer with TFA added as ion-pairing reagent. This buffer results in high column stability [66], excellent reproducibility and low silanol interactions and, in addition, gives the possibility of manipulating the selectivity by altering both the pH and the TFA concentration. Desalting can also be made with a solid-phase extraction column. This choice of buffer is determined by the practical restrictions put on mobile phase and it spans most of the variations that are possible within these restrictions. In addition, selectivity changes have been observed with the proposed variations in organic modifier [4,79,80], TFA concentration [4,64,81,82] and pH [83,84]. Note that in this work pH is limited to two discrete values, namely 2.8 and 6.5. This is due to the low column stability outside this range and the low buffer capacity in the intermediate range. Clearly, these limitations are not absolute, as both column stability and buffer capacity change gradually with pH. However, considering the necessity for reproducibility, the consequence of column leakage and the large sample loads commonly utilized, only very small deviations from these pH values can be recommended.

It should be stated that the buffer suggested in this work is a compromise. The main disadvantage with this buffer is that it is more complicated to desalt than the more common mobile phase systems with only TFA. The demands are

different for different situations, and the mobile phase selection should always be determined by the application. For example, in separations prior to radioimmunological assay, formic acid could well be used as buffer. UV detection is then only used for the determination of the retention volume for standards, making baseline drift a minor problem.

The low UV absorbance and viscosity of acetonitrile make it the most commonly used organic modifier for peptide separations. By using other organic modifiers different selectivity can be obtained, although the selection is strongly limited by the detection wavelength. The most popular alternative for acetonitrile is propanol, owing to its low UV absorbance, high elution strength and a different selectivity compared with acetonitrile [6,9,81,85,86]. In this work, an acetonitrile–2-propanol mixture was used as the alternative to acetonitrile. This mixture was chosen as a compromise between selectivity and efficiency, as pure 2-propanol has a high viscosity, giving low chromatographic efficiency [4].

The hybrid strategy

The strategy proposed in this work can be described as a hybrid strategy, which means that retention modelling is combined with direct response optimization. An experimental design is made for the mobile phase systems that are considered. The experimental design is illustrated in Fig. 4 and the set values are listed in Table I. The design is a full factorial design, where all combinations of variable settings are considered. By adopting this design, possible synergistic effects can be acknowledged. For each mobile phase system, represented by the corners of the cube in Fig. 4, three gradients with different slopes are run. As explained above, the three gradients are then used to calculate how fast a gradient can be run, with the specific mobile phase system, so that the desired resolution is still obtained. The maximum steepness is called the response for this mobile phase system.

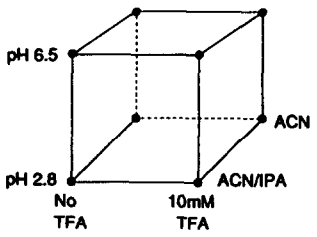

Fig. 4. Experimental design of the mobile phase system. Each corner represents a combination of the set values of the mobile phase variables in Table I. ACN = acetonitrile; IPA = 2-propanol.

TABLE I

MOBILE PHASE VARIABLES USED IN OPTIMIZATION

Variable	Lower value	Upper value
pH	2.8	6.5
TFA concentration (m*M*)	0	10
Organic modifier	Acetonitrile	Acetonitrile–2-propanol (60:40)

The optimum mobile phase system is taken as the one that allows the highest gradient slope. It is important to note that no model is made of either response or retention as a function of the mobile phase variables. The optimization of the mobile phase system is a primitive grid search, based on reasons discussed in a later section.

An example

The strategy described above was applied to the separation of angiotensin II, which was added to a tryptic digest of myoglobin, serving as the matrix. It is important to note that the matrix was considered as unknown. The mobile phase variables presented earlier, and illustrated in Fig. 4, were used. The range of slopes for the three gradient runs was 0.50–1.00%/ml, based on scouting experiments. The gradient slopes were kept low to ensure a high peak capacity which simplifies peak tracking. It has also been shown that extrapolation to faster gradients is more accurate than the opposite [47]. Calibration gradients can therefore be made with slopes lower than the expected optimum. The narrow range of gradient slopes also aids peak tracking as no large selectivity change can be expected over a small range. Extrapolation is, however, inherently more sensitive to experimental error

than interpolation, and care should be taken to minimize those errors [47]. Detection was made with a dual-wavelength detector, which combines high sensitivity with the possibility of using wavelength ratios for peak tracking.

For this application, the resolution required was set to 1.5. The responses, gradient slopes in %/min, are presented in Fig. 5, where the layout of mobile phase systems is the same as in Fig. 4. It is clear that large variations in response are obtained, hence an optimization can result in a major improvement in separation. It can also be seen that the variables are synergistic, *e.g.*, the result at low pH is highly dependent on the setting of the other variables. Using an experimental design is necessary; varying one variable at a time can be misleading.

As can be seen from Fig. 5, the fastest gradient, $b = 0.37$, will be obtained at pH 6.5 with 10 mmol/l TFA and pure acetonitrile as organic modifier. This steepness corresponds to a slope of 2.4%/ml. This is surprisingly fast considering the complexity of the sample. With one of the mobile phases systems, all interferences could not be tracked between the various gradients. The response is then based on the interferences that actually could be tracked. This is not a problem as long as all peaks have been tracked for the mobile phase system that yielded the highest response. In addition, in cases where peak tracking is difficult, there are usually a large number of interferences, making it unlikely that a high response can be obtained. An enlargement of the predicted and actual chromatograms with this mobile phase system (Fig. 6) shows the angiotensin II peak and its potential interferences. The chromatograms match well, despite a large degree of extrapolation of gradient slope. In Fig. 7 a larger portion of the actual chromatogram is shown. The resolution is slightly lower than the predicted value owing to some tailing and interfering peaks smaller than the 2% of the angiotensin II peak. It would, of course, be desirable to have the peptides of interest separated from all interfering peaks. This is almost impossible as for any biological sample there will be hundreds of small interfering peaks. The number of interfering peaks typically increases exponentially as the limit of concern is lowered. The lowest possible limit of concern is set by instrumental noise, but in practice the limit has to be higher. The consequence is that totally pure peaks will never be

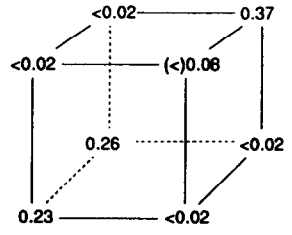

Fig. 5. Responses or the fastest gradient steepness giving the desired solution. The symbol < means that the required resolution is obtained at a gradient steepness lower than that shown. The symbol (<) indicates that not all peaks were tracked but with the peaks that were tracked the desired resolution was obtained at the specified steepness.

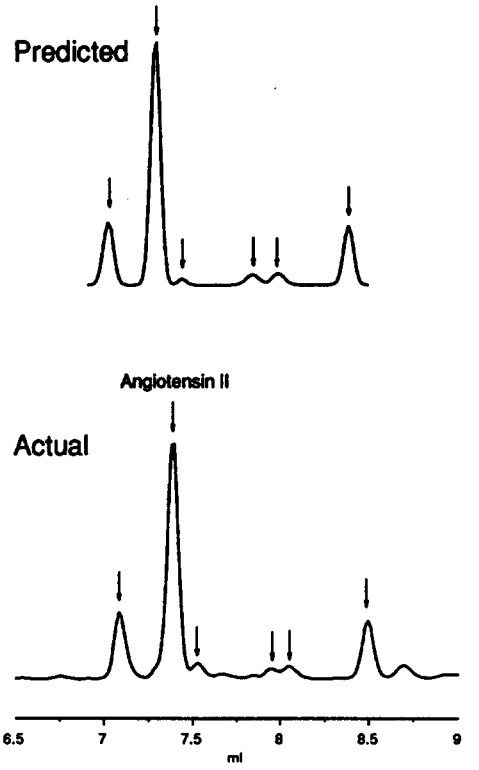

Fig. 6. Predicted and actual chromatograms with the fastest gradient giving the desired resolution with the optimum mobile phase system, *i.e.*, pH 6.5, acetonitrile as organic modifier and TFA added. Peaks that were considered are marked with arrows.

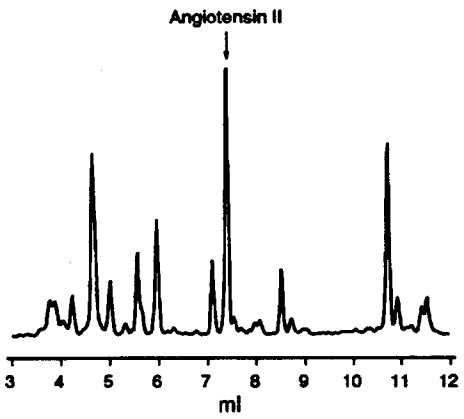

Fig. 7. Actual chromatogram obtained with a gradient starting at 10% of organic modifier.

obtained in separations of complex matrices. In the optimization of a separation it is also meaningless to seek separation from extremely small interferences as there is a natural variation over samples and the contents of these small interferences are likely to vary.

Number of experiments

The number of experiments needed in this strategy is dependent on how many mobile phase variables are considered. In the application presented, three variables were used and 25 experiments were made (three gradient for each mobile phase system and one run under the optimum conditions). It was pointed out earlier that the choice of mobile phase systems is related to the specific application. The number of experiments will be a function of the mobile phase system that is relevant and how many variables one can, and wants to, use. A large number of experiments might be discouraging, but working with a structured strategy the experimental work can usually be made very efficient. It is also our experience that with automated instrumentation the most time-consuming part is the evaluation of the chromatograms and not the experimental work.

Separation of several peptides

The proposed strategy is illustrated here with the separation of one peptide from a complex background. This can easily be extended to several peptides and the aim will then be to obtain a minimum resolution for all peptides of interest with the fastest possible gradient. The limitation of this strategy is that only non-segmented gradients are used.

Extensive use of models

It is tempting to extend the use of retention models, or to use response models, for the prediction of the fastest gradient for an intermediate mobile phase system. There are, however, several reasons for not following this approach. As been pointed out earlier, the pH range is highly restricted. This exclusion of significant variations in pH, which is the most powerful variable, leaves concentration of TFA and composition of organic modifier as quantitative variables. It is possible to make response models for these variables, but models of response are often unsmooth, creating a need for higher order terms and many experiments. The other alternative, *i.e.*, retention models, generally needs quadratic terms, which will increase the number of experiments. Peak tracking can also be difficult when alterations are made to the mobile phase. Finally, it is our experience that with a set pH, the concentration of ion-pairing reagent and the type of organic modifier have no dramatic effect on selectivity, hence fine tuning is rarely meaningful.

CONCLUSIONS

The proposed strategy for optimization of the separation of one peptide from a complex, unknown matrix has been proved to work with a real example. The combination of the predictive capability of gradient theory with experimental design is shown to be extremely powerful. Peak tracking, the weak point in many optimization strategies, is performed to an extent that is realistic for real samples. The selection of mobile phase variables, the initial step in optimization, is crucial in peptide separations by reversed-phase liquid chromatography. This selection is always a compromise, which is dependent on the application. The compromise used in this work

gave acceptable results in terms of peak shape, reproducibility and column stability.

The strategy presented in this work is applicable to a wide range of complex peptide samples. It will hopefully make method development more efficient and eliminate the common trial-and-error approach.

SYMBOLS

φ	Concentration of organic modifier (%)
φ_0	Starting concentration of organic modifier (%)
a	Model parameter
B	Gradient slope (%/ml)
b	Gradient steepness
F	Flow-rate (ml/min)
k'_0	k' at starting concentration of organic modifier
k'_f	k' when the solute leaves the column
m	Model parameter ($\%^{-1}$)
N	Plate number
R_s	Resolution
s	Gradient rate (%/min)
V_g	Retention volume (ml)
V_m	Dead volume (ml)
W_{bg}	Peak width at base (ml)

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REFERENCES

1 W.S. Hancock, C.A. Bishop and M.T.W. Hearn, *Science*, 200 (1978) 1168.
2 W.S. Hancock, C.A. Bishop and M.T.W. Hearn, *FEBS Lett.*, 72 (1976) 139.
3 K. Benedek, S. Dong and B.L. Karger, *J. Chromatogr.*, 317 (1984) 227.
4 K.D. Nugent, W.G. Burton, T.K. Slattery, B.F. Johnson and L.R. Snyder, *J. Chromatogr.*, 443 (1988) 381.
5 G. Thévenon and F.E. Regnier, *J. Chromatogr.*, 476 (1989) 499.
6 C.T. Wehr, R.P. Lundgard and K.D. Nugent, *LC·GC Int.*, 2(3) (1989) 24.
7 J.D. Pearson and F.E. Regnier, *J. Liq. Chromatogr.*, 6 (1983) 497.
8 W.G. Burton, K.D. Nugent, T.K. Slattery, B.R. Summers and L.R. Snyder, *J. Chromatogr.*, 443 (1988) 363.
9 K.A. Cohen, K. Schellenberg, K. Benedek, B.L. Karger, B. Grego and M.T.W. Hearn, *Anal. Biochem.*, 140 (1984) 223.
10 J.L. Glajch, J.J. Kirkland and J. Köhler, *J. Chromatogr.*, 384 (1987) 81.
11 N. Tanaka, K. Kimata, Y. Mikawa, K. Hosoya, T. Araki, Y. Ohtsu, Y. Shiojima, R. Tsuboi and H. Tsuchiya, *J. Chromatogr.*, 535 (1990) 13.
12 D.S. Stegehuis, U.R. Tjaden, C.M.B. van den Beld and J. van der Greef, *J. Chromatogr.*, 549 (1991) 185.
13 R. Newcomb, *LC·GC Int.*, 2(9) (1989) 28.
14 C.E. Goewie, *J. Liq. Chromatogr.*, 9 (1986) 1431.
15 A. Drouen, J.W. Dolan, L.R. Snyder, A. Poile and P.J. Schoenmakers, *LC·GC Int.*, 5(2) (1992) 28.
16 P.J. Schoenmakers, *Optimization of Chromatographic Selectivity*, Elsevier, Amsterdam, 1986.
17 J.C. Berridge, *Techniques for the Automated Optimization of HPLC Separation*, Wiley, Chichester, 1985.
18 I. Molnar, R. Boysen and P. Jekow, *J. Chromatogr.*, 485 (1989) 569.
19 J.W. Dolan, *LC·GC Int.*, 3(7) (1990) 17.
20 J.L. Glajch, M.A. Quarry, J.F. Vasta and L.R. Snyder, *Anal. Chem.*, 58 (1986) 280.
21 H.J. Issaq and K.L. McNitt, *J. Liq. Chromatogr.*, 5 (1982) 1771.
22 J.K. Strasters, H.A.H. Billiet, L. de Galan and B.G.M. Vandeginste, *J. Chromatogr.*, 499 (1990) 499.
23 E.P. Lankmayr, W. Wegscheider, J. Daniel-Ivad, I. Kolossváry, G. Csonka and M. Otto, *J. Chromatogr.*, 485 (1989) 557.
24 H.A.H. Billiet and L. de Galan, *J. Chromatogr.*, 485 (1989) 27.
25 J.L. Glajch and J.J. Kirkland, *J. Chromatogr.*, 485 (1989) 51.
26 J.C. Berridge, *J. Chromatogr.*, 244 (1982) 1.
27 A.F. Fell, T.A.G. Noctor, J.E. Mama and B.J. Clark, *J. Chromatogr.*, 434 (1988) 377.
28 P.J. Schoenmakers and T. Blaffert, *J. Chromatogr.*, 384 (1987) 117.
29 P.M.J. Coenegracht, A.K. Smilde and A. Knevelman, *J. Liq. Chromatogr.*, 12 (1989) 77.
30 J.M. Juárez, A. Bermejo, J.L. Bernal, M.J. del Nozal and G.A. García, *Chromatographia*, 29 (1990) 338.
31 Y. Hu and D.L. Massart, *J. Chromatogr.*, 485 (1989) 311.
32 E.P. Lankmayr, W. Wegscheider, J.C. Gfeller, N.M. Djordjevic and B. Schreiber, *J. Chromatogr.*, 485 (1989) 183.
33 H.J.G. Debets, B.L. Bajema and D.A. Doornbos, *Anal. Chim. Acta*, 151 (183) 131.
34 A. Peeters, L. Buydens, D.L. Massart and P.J. Schoenmakers, *Chromatographia*, 26 (1988) 101.
35 B.G.M. Vandeginste, G. Kateman, J.K. Strasters, H.A.H. Billiet and L. de Galan, *Chromatographia*, 24 (1987) 127.

36 G.G.R. Seaton and A.F. Fell, *Chromatographia*, 24 (1987) 208.
37 M. Maeder, *Anal. Chem.*, 59 (1987) 527.
38 A.G. Wright, A.F. Fell and J.C. Berridge, *J. Chromatogr.*, 458 (1988) 335.
39 J.K. Strasters, F. Coolsaet, A. Bartha, H.A.H. Billiet and L. de Galan, *J. Chromatogr.*, 499 (1990) 523.
40 T.P. Bridge, M.H. Williams and A.F. Fell, *J. Liq. Chromatogr.*, 12 (1989) 23.
41 R.J. Lynn, S.D. Patterson and R.E.A. Escott, *LC·GC Int.*, 3(9) (1990) 54.
42 M.A. Stadelius, H.S. Gold and L.R. Snyder, *J. Chromatogr.*, 296 (1984) 31.
43 B.F.D. Ghrist and L.R. Snyder, *J. Chromatogr.*, 459 (1988) 43.
44 J.W. Dolan, D.C. Lommen and L.R. Snyder, *J. Chromatogr.*, 485 (1989) 91.
45 J. Schmidt, *J. Chromatogr.*, 485 (1989) 421.
46 S. Heinisch, J.-L. Rocca and M. Kolosky, *Chromatographia*, 29 (1990) 482.
47 N. Lundell, *J. Chromatogr.*, 639 (1993) 97.
48 L.R. Snyder, in Cs. Horváth (Editor), *High Performance Liquid Chromatography: Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 207.
49 L.R. Snyder and M.A. Stadalius, in Cs. Horváth (Editor), *High Performance Liquid Chromatography: Advancaes and Perspectives*, Vol. 4, Academic Press, New York, 1986, p. 195.
50 P. Jandera and J. Churáček, *Gradient Elution in Column Liquid Chromatography, Theory and Practice*, Elsevier, Amsterdam, 1985.
51 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 223.
52 M.T.W. Hearn and B. Grego, *J. Chromatogr.*, 255 (1983) 125.
53 M.J. O'Hare, M.W. Capp, E.C. Nice, N.H.C. Cooke and B.G. Archer, *Anal. Biochem.*, 126 (1982) 17.
54 P.S. Sadek, P.W. Carr, L.D. Bowers and L.C. Haddad, *Anal. Biochem.*, 153 (1986) 359.
55 K.E. Bij, C. Horváth, W.R. Melander and A. Nahum, *J. Chromatogr.*, 203 (1981) 65.
56 S. Linde and B. Welinder, *J. Chromatogr.*, 536 (1991) 43.
57 J.E. Rivier, *J. Chromatogr.*, 202 (1980) 211.
58 W.S. Hancock and J.T. Sparrow, *J. Chromatogr.*, 206 (1981) 71.
59 C.T. Mant and R.S. Hodges, *Chromatographia*, 24 (1987) 805.
60 W.S. Hancock, C.A. Bishop, R.L. Prestidge, D.R.K. Harding and M.T.W. Hearn, *J. Chromatogr.*, 153 (1978) 391.
61 W.S. Hancock, C.A. Bishop, R.L. Prestidge and M.T.W. Hearn, *Anal. Biochem.*, 89 (1978) 203.
62 J.E. Rivier, *J. Liq. Chromatogr.*, 1 (1978) 343.
63 H.P.J. Bennett, C.A. Browne and S. Solomon, *J. Liq. Chromatogr.*, 3 (1980) 1353.
64 D. Guo, C.T. Mant and R.S. Hodges, *J. Chromatogr.*, 386 (1987) 205.
65 A.N. Starratt and M.E. Stevens, *J. Chromatogr.*, 194 (1980) 421.
66 Data available on request.
67 M.T.W. Hearn, in Cs. Horváth (Editor), *High Peformance Liquid Chromatography: Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 87.
68 J.A. Smith and M.J. O'Hare, *J. Chromatogr.*, 299 (1984) 13.
69 S. Linde and B.S. Welinder, *J. Chromatogr.*, 548 (1991) 195.
70 D.J. Poll and D.R.K. Harding, *J. Chromatogr.*, 469 (1989) 231.
71 B. Fransson, *J. Chromatogr.*, 361 (1986) 161.
72 C. Dewaele, P. Mussche and M. Verzele, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 616.
73 L.A. Witting, D.J. Gisch, R. Ludwig and R. Eksteen, *J. Chromatogr.*, 296 (1984) 97.
74 J.J. Kirkland, J.L. Glajch and R.D. Farlee, *Anal. Chem.*, 61 (1989) 2.
75 K. Larsson, W. Hermann, P. Möller and D. Sanchez, *J. Chromatogr.*, 450 (1988) 71.
76 O. Mikes and J. Coupek, in K.M. Gooding and F. Regnier (Editors), *HPLC of Biological Macromolecules: Methods and Applications*, Marcel Dekker, New York, 1990, p. 25.
77 T. Ohtani, Y. Tamura, M. Kasai, T. Uchida, Y. Yangihara and K. Noguchi, *J. Chromatogr.*, 515 (1990) 175.
78 K.A. Tweeten and T.N. Tweeten, *J. Chromatogr.*, 359 (1986) 111.
79 S.D. Power, M.A. Lochrie and R.O. Poyton, *J. Chromatogr.*, 266 (1983) 585.
80 C.T. Mant and R.S. Hodges, *J. Liq. Chromatogr.*, 12(1 & 2) (1989) 139.
81 H. Linder and W. Helliger, *Chromatographia*, 30 (1990) 518.
82 L.R. Gurley, D.A. Prentice, J.G. Vadez and W.D. Spall, *Anal. Biochem.*, 131 (1983) 465.
83 M. Andre, *J. Chromatogr.*, 351 (1986) 341.
84 W.H. Vensel, V.S. Fujita, G.E. Tarr, E. Margoliash and H. Kayser, *J. Chromatogr.*, 266 (1983) 491.
85 M. Rubinstein, *Anal. Biochem.*, 98 (1979) 1.
86 A.K. Taneja, S.Y.M. Lau and R.S. Hodges, *J. Chromatogr.*, 317 (1984) 1.