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UNATTENDED OPTIMISATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS USING THE MODIFIED SIMPLEX ALGORITHM

J. C. BERRIDGE

Pfizer Central Research, Sandwich, CT13 9NJ, Kent (Great Britain)

SUMMARY

It is shown that the modified sequential simplex algorithm can be used as the basis for the unattended optimisation of reversed-phase liquid chromatographic separations. A chromatographic response function is computed to evaluate individual chromatograms with respect to resolution and analysis time. This function is used automatically by a microprocessor-controlled chromatograph to optimise experimental parameters. The use of the procedure is illustrated by the automated optimisation of two and three component mobile phases and the optimisation of a gradient elution profile.

INTRODUCTION

The primary objective in the development of high-performance liquid chromatography (HPLC) separations is to optimise the chromatographic performance through the adjustment of experimental factors. This practical goal is the subject of increasing attention[1-9] with emphasis on the optimisation of column efficiency, solvent strength and selectivity. The optimisation of a separation will normally follow the selection, based on the chromatographer's experience and intuition, of vital parameters such as column length, stationary phase type and the principal components of the mobile phase. Solvent strength and selectivity are then optimised by adjustment of the mobile phase composition.

The advent of the microprocessor controlled HPLC has brought with it the full computer control of chromatographic parameters such as the mobile phase composition, gradient elution profile, mobile phase flow-rate, column temperature and the detector conditions. This instrumental control and the associated computational power means that, once the chromatographer has specified the constraints of the system and the initial experiments, it is possible to optimise chromatographic separations automatically without the need for further intervention by the chromatographer.

Reversed-phase separations using hydrocarbon bonded phases are probably the most versatile and popular of current HPLC methods. There are now a number of

procedures available for the systematic optimisation of such separations. These are based on theoretical models, developed to predict retention behaviour as a function of, for example, mobile phase composition or temperature[1–4,7–9], or use statistical or sequential search techniques[5,10–12].

The sequential simplex algorithm[13,14] is one of the most efficient, multidimensional, sequential search procedures for locating an experimental optimum and it has been used successfully in a wide variety of analytical situations (e.g., refs. 15–19). The detailed operation of the simplex procedure has been described by other workers[14,15,17]. In essence the algorithm directs the adjustment of experimental conditions away from those which give a poor result, or response, towards conditions which give a more favourable response. In order to be able to use the procedure for chromatographic optimisation some means of ordering the experiments in relation to the quality of the separation is required. It is convenient to describe the quality of a given separation by a simple, numeric term which can then be used as the response in the simplex procedure.

The concept of a Chromatographic Response Function (CRF)[20] provides a numerical description of the separation quality which may be used as the input to the simplex optimisation procedure. The CRF was originally described as a function of the peak separation, P_i, between adjacent pairs of peaks:

$$CRF = \Sigma \ln P_i \tag{1}$$

It was then extended by other workers[5] to include a comparison of the actual separation, P_i, with a desired separation, P_0, and the actual analysis time, T_L, with an acceptable time, T_M, for analysis:

$$CRF = \Sigma f(P_i, P_0) + g(T_L, T_M) \tag{2}$$

In the present work this concept was extended to include more information about the quality of the separation.

The ability to quantify the quality of a separation, using a simple numerical response function, and to search for a maximum value has been shown to provide a powerful means for optimising liquid chromatographic separations. The procedure has been applied to the optimisation of isocratic mobile phase compositions[11,21] and to the optimisation of gradient elution profiles[5,22]. In these cases the chromatographer was required to make a significant contribution to the experimental procedure, particularly in the setting up of new experiments.

The need for intervention by the chromatographer can make the application of the simplex procedure laborious and time consuming. These limitations are removed totally if the procedure is operated by a computer controlled liquid chromatograph since manual intervention is no longer required. The extra time that may be required to set up an automated system such as is described will be easily recovered by the repeated use of the system once it is operating.

The unattended optimisation of gradient elution parameters has been the subject of a preliminary communication[23]. The aim of the present work was to extend the possibilities of automatic method development and to use the simplex algorithm as part of computer programs to carry out the unattended optimisation of: (a) isocratic

binary mobile phase composition and flow-rate; (b) binary gradient elution profile; and (c) ternary mobile phase composition.

EXPERIMENTAL

Materials

The separations were carried out using stainless steel columns (10 × 0.5 cm; Shandon Southern, Runcorn, Great Britain) packed with LiChrosorb RP-18 with a particle diameter of 5 μm (E. Merck, Darmstadt, G.F.R.) or Hypersil ODS with a particle diameter of 5 μm (Shandon Southern). Mobile phases were prepared from HPLC grade methanol and acetonitrile (Rathburn Chemicals, Walkerburn, Great Britain) and glass-distilled demineralised water. Glacial acetic acid (BDH, Poole, Great Britain) and concentrated ammonia solution (BDH) were used for pH adjustment. All mobile phases were deaerated by stirring under vacuum before use. Separations were conducted at room temperature.

All solutes were reagent grade and were used without purification. They were dissolved in methanol–water (70:30) prior to injection.

Equipment

An Analyst 7800 liquid chromatograph (Laboratory Data Control, Stone, Great Britain) was used, having two pumps (Constametric III), a variable wavelength ultraviolet detector (Spectromonitor III) and a 60-position automatic sampling system with a 20-μl pneumatically operated injection valve (Rheodyne). The chromatograph was controlled by a Chromatography Control Module (CCM; Laboratory Data Control) equipped with Version B firmware, a BASIC interpreter and 32K of random access memory.

Where a ternary mobile phase was required, a Constametric IIG pump (Laboratory Data Control) was added to the system. This pump was controlled by one of the two analogue outputs provided on the HPLC Interface Unit of the CCM.

Calculation of Chromatographic Response Function

The form of the CRF was modified from that of Watson and Carr[5] to provide a more flexible function to allow time or resolution criteria to be controlled more precisely. The specific form of the CRF used is given as eqn. 3:

$$CRF = \sum_{i=1}^{L} R_i + L^x - a|T_M - T_L| - b(T_0 - T_1) \tag{3}$$

R_i is the resolution between adjacent pairs of peaks, in practice limited to a maximum value of 2 so that all pairs of peaks that exceed this resolution make no further contribution to the CRF. The total number of peaks detected, L, is weighted with an exponent, x, such that the detection of the maximum number of peaks can be made the most important requirement; this may be at the expense of only partial resolution between some adjacent pairs. The modulus of the difference, in minutes, between an acceptable analysis time, T_M, and the retention time of the last eluted peak, T_L, is weighted by an arbitrary factor, a. The use of the absolute value and a suitable weighting factor permits the precise positioning of the last eluted peak if this is

considered desirable. The final term of eqn. 3 reduces the CRF if the first peak, T_1, is eluted before a specified minimum retention time, T_0: b is an arbitrary weighting factor. Values of a, b and x in the range 0.5–2.0 were used in the present work. The chromatographic response function of eqn. 3 is designed to increase as the optimum is approached.

In order to calculate the chromatographic response function a rapid method of estimating resolution was required. The resolution, R, between two peaks can be expressed as[1]

$$R = 2(t_2 - t_1)/(w_1 + w_2) \tag{4}$$

where t is retention time and w is peak width at baseline.

The chromatographic plate count, N, for symmetrical, Gaussian shaped peaks can be determined using eqns. 5 or 6[24]

$$16(t/w)^2 \tag{5}$$

$$2(ht/A)^2 \tag{6}$$

where h is the peak height and A the area. Combining eqns. 5 and 6 gives an estimate of the peak base width:

$$w = 4A/(2\pi)^{0.5}h \tag{7}$$

In practice chromatographic peaks are rarely Gaussian or symmetrical and eqn. 7 will underestimate peak base width. Eqn. 7 was therefore reduced to the form shown as eqn. 8 to account for peak asymmetry:

$$w = 2A/h \tag{8}$$

By scaling the integrator outputs of peak retention times, heights and areas to self consistent units, peak base widths and hence resolution were estimated with minimum computation. It must be pointed out that the use of this method is dependent upon the ability of the integrator used to make "intelligent" decisions about the nature of the chromatographic peaks with which it is dealing. If the ratios of peak heights become large the integrator may be unable to discriminate between peaks, or the calculation of heights and areas may be in error. Despite these limitations the proposed method is not felt to be restrictive when carrying out separation optimisation.

Programming the Chromatography Control Module

The control of the chromatograph is through a series of files which contain all the chromatographic and integration parameters and which control the sequence of injections of the autosampler. All the files are accessible by programs written in BASIC and any of the parameters contained within them may be changed by such programs.

Three programs were written to carry out the optimisation procedures referred to above. These programs have been given the following titles:

(a) ISOOPT the optimisation of an isocratic binary mobile phase composition and flow-rate

(b) GRADOPT the optimisation of gradient elution profiles

(c) TERNOPT the optimisation of ternary mobile phase composition

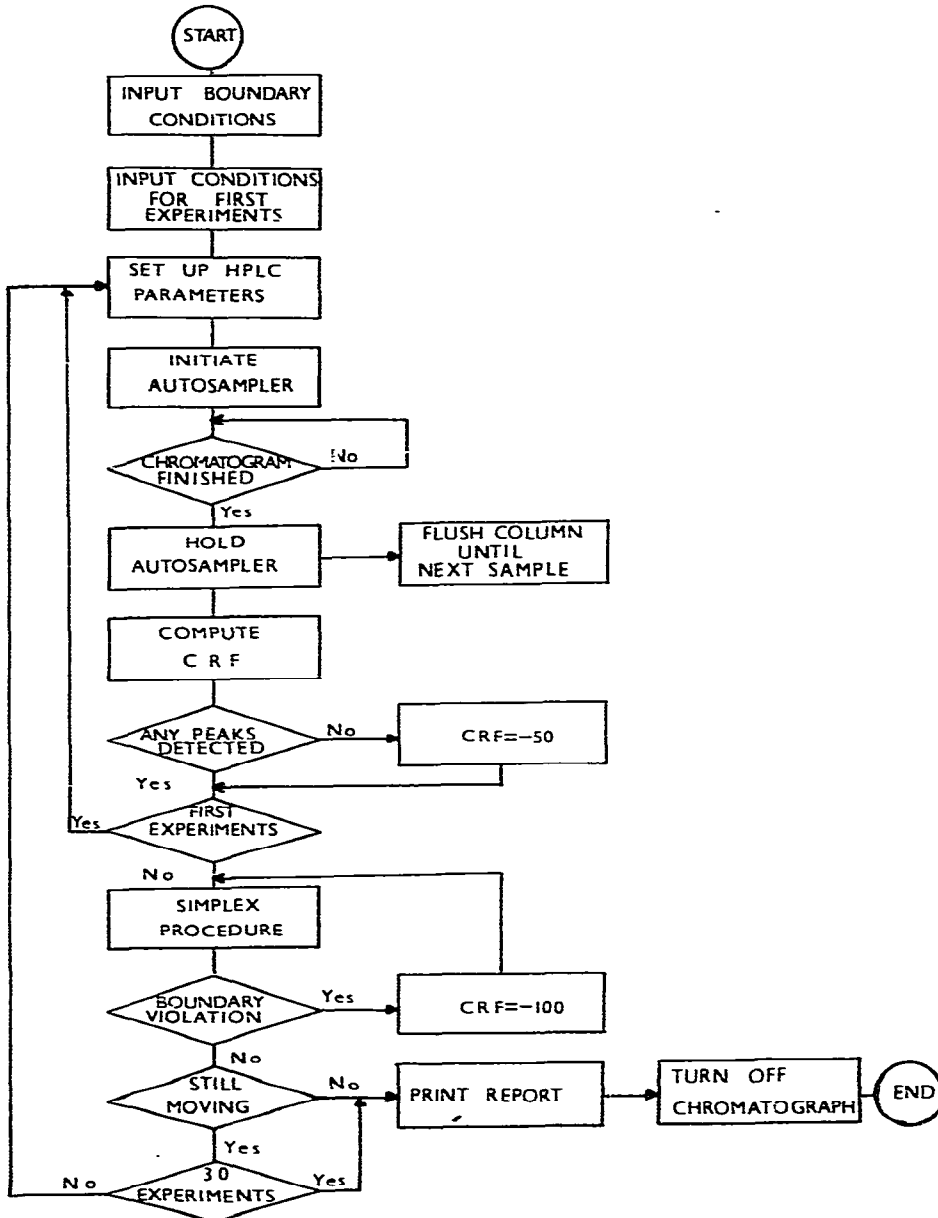All three programs incorporated the modified simplex algorithm of Nelder and

Fig. 1. Block diagram of TERNOPT optimisation program.

Mead[25]. This modification causes the simplex to contract away from regions of unfavourable response and to expand in the direction of a more favourable response. A simplified block structure of the program TERNOPT is shown in Fig. 1: all three programs had the same essential structure.

A check was built into the programs to determine whether the simplexes are still making significant moves in the variable ranges under investigation. It was found that if none of the variables changed by more than 3 % of its range in $n + 1$ experiments, where n is the number of variables, then the procedure had located an optimum and further experiments would not give any improvement in the separations. The number of experiments conducted during each optimisation procedure was restricted to 30. The need for restriction arises from limitations in memory capacity such that parameters and results from 30 experiments only could be stored. This limitation was not found to cause any practical problems since, if an optimimum had not been found by experiment 30. the simplex had probably failed. The most common cause of failure was not in the logic of the procedure but as a consequence of noisy chromatographic data: in such cases the simplex would be directed away from the true optimum by a CRF made falsely high as a result of the noise being interpreted as real peaks. The misdirection of the simplex by falsely high CRF values was largely compensated for by the built in check of the "$n + 1$" rule[16] which requires that a persistent vertex be re-evaluated following retention in $n + 1$ simplexes. Occasionally, where excessive noise did occur, the procedure was halted and restarted when more stable conditions had been established.

Where a third pump was required (as in TERNOPT) its flow was controlled through a 0–5 V d.c. signal generated from a spare analogue output available on the HPLC Interface Unit of the CCM. The required flow-rate was converted to a two byte binary number and written to the appropriate address of the digital to analogue converter.

RESULTS AND DISCUSSION

The use of the three optimisation programs will be discussed using an example for each case.

ISOOPT: the separation of 2-substituted pyridines
A mixture was prepared containing 2-aminopyridine, 2-formamido-3-methylpyridine, 2-amino-5-methylpyridine and 2-ethylpyridine. Since ISOOPT considers only two variables, namely the proportion of the stronger eluting solvent and the mobile phase flow-rate, only three initial experiments were required. The positions of these experiments within the boundary constraints were located according to the method described by Yarbro and Deming[26] for a large step size simplex. The experimental parameters and boundary conditions are shown in Table I. The requested analysis time was 8 min, with each chromatographic run limited to 12 min. The precise form of the CRF used was:

$$CRF = \sum_{i=1}^{L} P_i + L^{1.5} - 1.5 | 8 - T_L | - 1.0 (1 - T_1) \qquad (9)$$

TABLE I

EXPERIMENTAL PARAMETERS USED FOR ISOOPT

Column packing: Hypersil ODS. Solvents: A, water + 0.1 % ammonia; B, acetonitrile + 0.1 % ammonia. Detection: ultraviolet spectrophotometric at 240 nm.

Boundary conditions	Minimum	Maximum	Experiment No.		
			1	2	3
Flow-rate (ml min^{-1})	0.5	3.5	3.5	3.2	0.8
% B	0	99.9	0	90	10

The weightings for analysis time and the total number of peaks present were both set at 1.5 such that time considerations did not dominate the optimisation and so that resolution could be maximised.

The progress of the simplex in the composition/flow domain is shown in Fig. 2. Fig. 3 shows the CRF as a function of the experiment number and it can be seen that many of the early moves of the simplex were to conditions outside the preselected boundaries (experiments 4, 6, 8, 10 and 12). A response of − 100 was arbitrarily assigned to these experiments to force the simplex back inside its bounds. During the optimisation, experiment 17 gave a high response due to the detection of noise on the chromatographic signal. The vertex corresponding to this experiment was retained in the next three moves of the simplex and then rechecked according to the rules of the procedure. The response at this vertex was replaced with the averaged value.

Reference to Fig. 2 indicates an optimum at approximately 16% acetonitrile and a flow-rate of 1.4 ml min^{-1}. Fig. 4 shows the chromatogram obtained using these conditions. The retention time of the last eluted peak is 9.7 min, against a requested
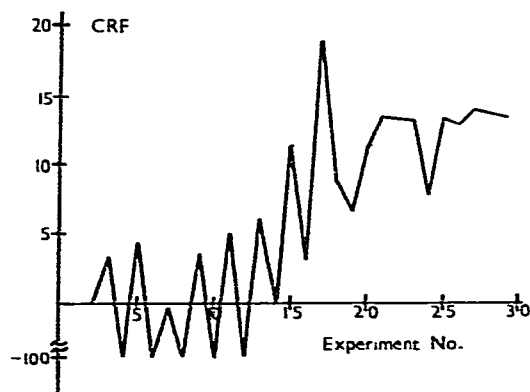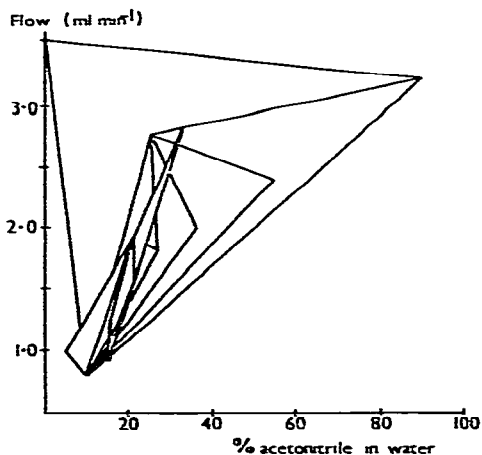

Fig. 2. Simplex optimisation using ISOOPT.

Fig. 3. Relationship of Chromatographic Response Function (CRF) with experiment numbers during optimisation with ISOOPT.
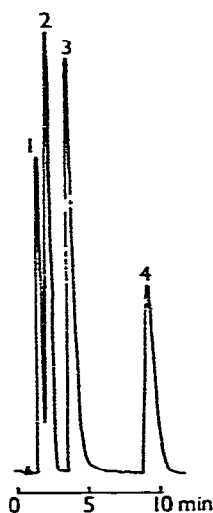
Fig. 4. Optimised isocratic separation of substituted pyridines: 10-min time constraint. Eluent: acetonitrile–water–ammonia (16:84:0.1), flow-rate 1.4 ml min^{-1}. Elution order: 1 = 2-aminopyridine; 2 = 2-formamido-3-methylpyridine; 3 = 2-amino-5-methylpyridine; 4 = 2-ethylpyridine.

time of 8 min. The analysis time is extended since time criteria were less heavily weighted than those of resolution.

Inspection of Fig. 2 indicates that the simplex quickly "homes in" on an approximate mobile phase composition (*ca.* 20 % acetonitrile) and then the major movements are in the selection of flow-rate. This is not unexpected since under isocratic conditions the mobile phase flow-rate should have only a minor effect on resolution, through its influence on plate height, but a major effect upon analysis time[5]. Whilst this observation implies that the two variables may be optimised independently, the use of the simplex procedure in a program such as ISOOPT removes the need for the chromatographer to intervene with a decision as to a potential mobile phase composition and to subsequently optimise flow-rate to meet time requirements.

GRADOPT: the separation of phenolic antioxidants

A mixture of three antioxidants was prepared containing 2-*tert.*-butyl-*p*-methoxyphenol (BHA), 2,6-di-*tert.*-butyl-*p*-cresol (BHT) and propyl gallate. The experimental parameters and boundary conditions used are shown in Table II. A linear gradient was optimised, the three variables being the initial percentage of the second solvent (solvent B), the final percentage of solvent B and the time taken to change between these values. Both solvents A and B (Table II) contained 5 % acetonitrile: this is a simple example of the generation of a ternary gradient from two solvent reservoirs[9]. The desired analysis time for the separation was set to 4 min, the calculation of the response factor being according to eqn. 10:

$$CRF = \sum_{i=1}^{L} P_i + L^2 - 2|4 - T_L| - 2.0\,(1.5 - T_1) \tag{10}$$

TABLE II

EXPERIMENTAL PARAMETERS USED FOR GRADOPT

Column packing: LiChrosorb RP-18. Solvents: A, water + 5% acetonitrile; B, methanol + 5% acetonitrile. Detection: ultraviolet spectrophotometric at 290 nm. Flow-rate: 2.0 ml min^{-1}.

Boundary conditions	Minimum	Maximum	Experiment No.			
			1	2	3	4
Initial % B	10	80	10	66	24	24
Gradient duration (min)	1	10	1	2.8	8.2	2.8
Final % B	40	99.9	99	89	89	50

The simplex algorithm used in ISOOPT was modified in accord with the proposals of Routh et al.[27] and Watson and Carr[5]: instead of rejecting experimental coordinates that violate boundary conditions, the simplex size was contracted to give a new experimental point located at the relevant boundary.

Fig. 5 shows the chromatographic response as a function of the experiment number. An optimum response was achieved rapidly and the optimisation concluded at experiment 15 because the response was changing by less than 2%. A chromatogram using the optimum conditions is presented as Fig. 6. Although only three peaks were expected the optimisation has found, and taken into account, a fourth, un-
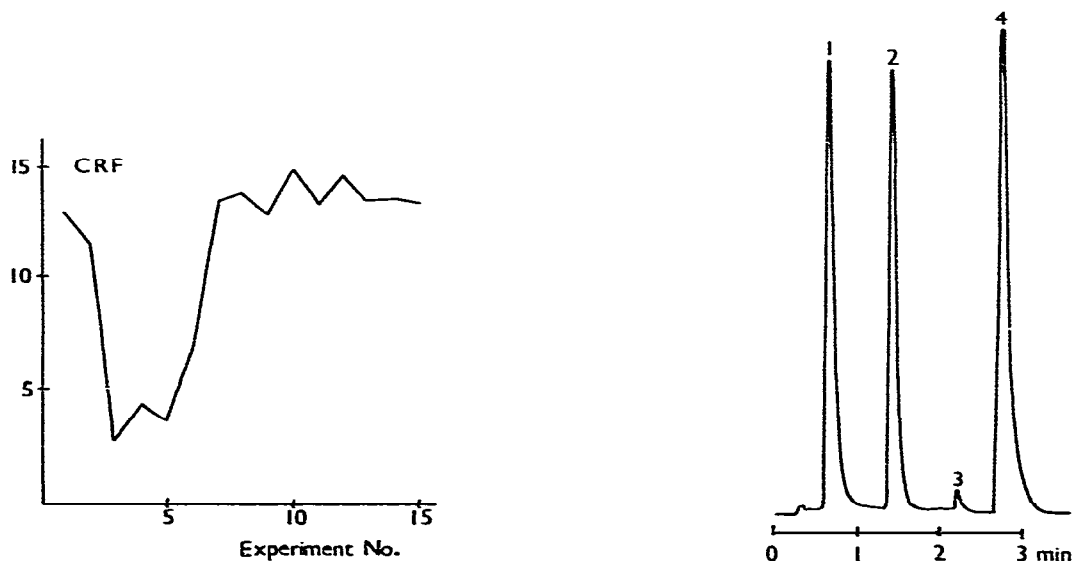

Fig. 5. Relationship of Chromatographic Response Function (CRF) with experiment number during optimisation with GRADOPT.

Fig. 6. Optimised gradient separation of antioxidants. Solvents: A, acetonitrile–water (5:95); B, acetonitrile–water (95:5). Linear gradient A–B (56:44) to 100% B in 1.5 min. Column: 10 cm × 5 mm I.D., 5-μm LiChrosorb C-18. Peaks: 1 = propyl gallate; 2 = BHA; 3 = unknown; 4 = BHT.
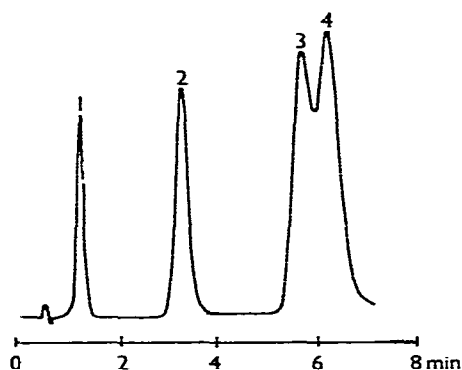
Fig. 7. Separation of substituted phenols before optimisation. Eluent: methanol–water–acetic acid (55:45:0.1). Elution order: 1 = methyl. 2 = propyl, 3 = butyl p-hydroxybenzoate, 4 = propyl gallate.

known peak which was not identified. One of the reasons for such rapid convergence on the optimum is that it is situated at one of the boundaries.

TERNOPT: the separation of substituted phenols

The use of ternary mobile phases in reversed-phase HPLC offers possibilities for great increases in the selectivity of separations; indeed ternary solvent systems may suffice for the majority of practical applications[9]. A theoretical model of ternary mobile phases in reversed-phase HPLC has been presented recently by Jandera *et al.*[9] for both gradient and isocratic separations. The program TERNOPT was developed to use the simplex procedure in the selection of isocratic ternary mobile phase compositions.

A mixture was prepared of three anti-microbial agents. methyl, propyl and butyl p-hydroxybenzoate and the antioxidant 2-*tert.*-butyl-p-methoxyphenol (BHA). It was not possible to separate the four components using an acidified mobile phase of methanol and water (Fig. 7) in the desired analysis time of 6 min. The program TERNOPT was used to optimise the separation using a ternary mobile phase. Table III lists the experimental parameters and boundary conditions used.

The requested analysis time was 6 min with a 10-min limitation on each chromatographic run. The CRF was calculated from eqn. 11:

TABLE III

EXPERIMENTAL PARAMETERS USED FOR TERNOPT

Column packing: Hypersil ODS. Solvents: A, water + 0.1 % acetic acid; B, methanol + 0.1 % acetic acid; C, acetonitrile + 0.1 % acetic acid. Detection: ultraviolet spectrophotometric at 235 nm. Flow-rate: 2.0 ml min^{-1}.

Boundary conditions	Minimum	Maximum	Experiment No.		
			1	2	3
% A	0	100	90	0	10
% B	0	100 − A	1	0	81
% C			9	100	9

$$CRF = \sum_{i=1}^{L} P_i + L^{1.5} - 1.5| 6 - T_L| - 1.0 (1 - T_1) \qquad (11)$$

The optimisation of a ternary mobile phase requires only two variables to describe the mobile phase composition: these may be the percentages of the first and second solvents, the third solvent proportion being the difference from 100% of the sum of the first two percentages. During the optimisation procedure, however, the boundary conditions for solvent B were recalculated at each experiment to take into account the proposed proportion of solvent A. Boundary violations were treated as in ISOOPT, an arbitrary value of − 100 being assigned to the CRF.

Results for TERNOPT are given in Table IV and the variation of CRF with experiment number is shown in Fig. 8. Experiments 4, 6, 8, 10 and 13 attempted to cross boundaries and were assigned a CRF of − 100. The vertex described by experiment 5 was persistent and was re-evaluated as experiment 12. Similarly the vertex of experiment 15 was re-evaluated as experiments 19 and 23.

TABLE IV

RESULTS OF THE OPTIMISATION OF TERNARY COMPOSITION USING TERNOPT

Experiment No.	% A	% B	% C	CRF	Average CRF*
1	90	1	9	−59	
2	0	0	100	−4.54	
3	10	81	9	−4.32	
4	−80	80	100	− 100	
5	47.5	20.7	31.8	14.91	
6	57.5	101.7	−59.2	− 100	
7	14.3	25.4	60.3	−4.26	
8	51.8	−34.9	83.1	− 100	
9	20.4	52	27.6	4.4	
10	53.6	47.3	−0.9	− 100	
11	24.1	30.8	45.1	4.48	
12	47.5	20.7	31.8	14.97	14.95
13	51.2	−0.5	49.3	− 100	
14	28.1	39.8	33.1	5.08	
15	51.5	28.7	19.8	15.99	
16	65.2	27.6	7.2	1.18	
17	70.9	10.6	18.5	0.06	
18	38.8	31.7	29.5	13.11	
19	51.5	28.7	19.8	16.07	16.04
20	60.2	17.7	22.1	6.14	
21	44.1	28.2	27.7	14.46	
22	54.9	21.2	23.9	16.01	
23	51.5	28.7	19.8	16.04	16.04
24	58.9	29.2	11.9	5.09	
25	50.3	22.8	26.9	16.64	
26	46.9	30.3	22.8	15.5	
27	52.9	23.4	23.7	16.78	
28	51.7	17.5	30.8	16.59	
29	51.6	20.3	28.1	17.18	

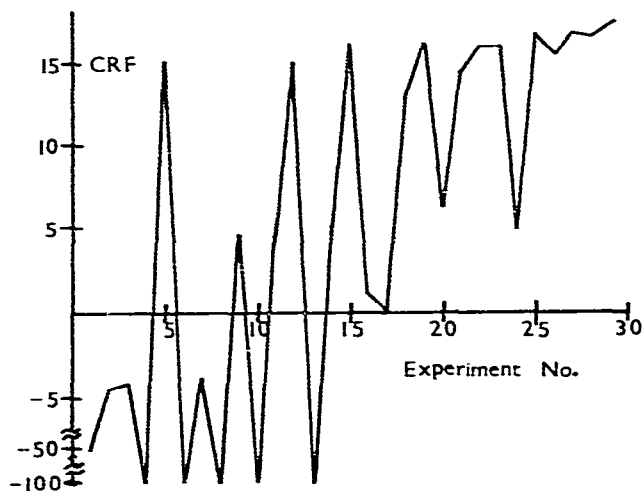* Weighted average response assigned to persistent vertices.

Fig. 8. Relationship of Chromatographic Response Function (CRF) with experiment number during optimisation with TERNOPT.

The progress of the simplex is shown as Fig. 9: the optimum composition being 52% water, 21% methanol and 27% acetonitrile. Fig. 10 shows the chromatogram of the mixture using these conditions. The retention time for the last eluted peak is 6.1 min and it is fully resolved from its predecessor.
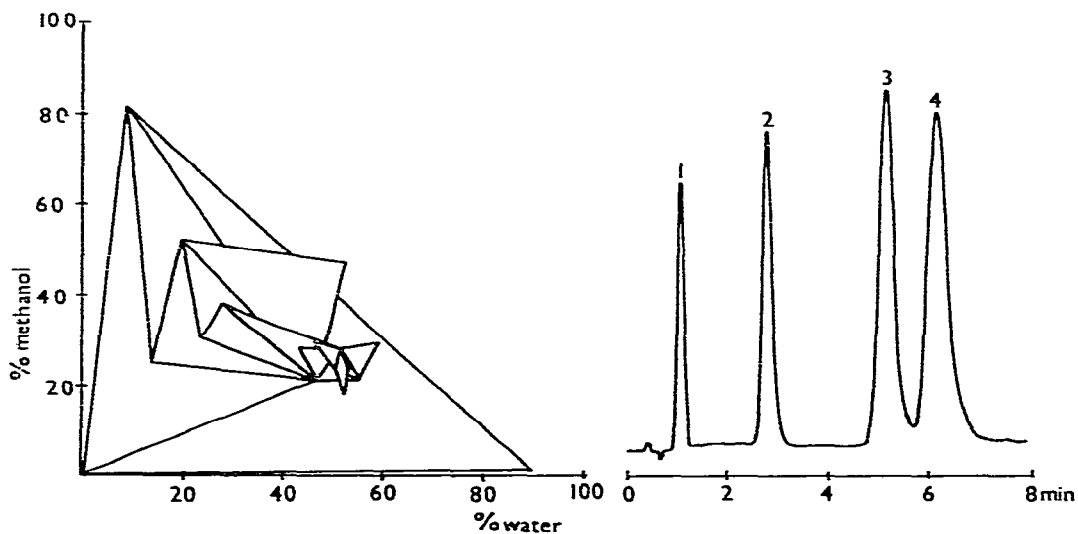

Fig. 9. Simplex optimisation using TERNOPT.

Fig. 10. Separation of substituted phenols optimised using TERNOPT. Eluent: methanol–acetonitrile–water–acetic acid (21:27:52:0.1). Elution order as in Fig. 7.

CONCLUSIONS

Three examples have been given of how the simplex procedure can be used in the automated optimisation of chromatographic separations. By using the simplex algorithm with a computer controlled chromatograph the totally unattended optimisation of reversed-phase separations has been demonstrated to be a practical reality.

It must be remembered that the simplex procedure, whilst being a very powerful means of locating an optimum response, will only optimise the local region and may not be useful for multimodal situations. It cannot guarantee finding the overall optimum chromatographic separation; there may exist several local maxima. This will inevitably be so if the elution order of peaks changes during the optimisation procedure although this phenomenon was not observed in the present work.

When dealing with an unknown mixture it is recommended that the procedure is repeated by restarting the simplex in a different region of the factor space in order to increase confidence in the quality of the optimum located. Previously, this would have greatly extended the time required of the chromatographer to optimise the separation; with an automated system this is no longer the case.

It may also happen that unrealistic goals, or over-constraining conditions, are set in which case there may not be a true optimum. The ability to adjust the time, resolution and peak weightings offers the chromatographer the flexibility to investigate different criteria, again with the minimum expenditure of personal time.

Finally, the optimum response located may be only a local optimum; there may be co-elution of bands that is not detected by the standard integration techniques. With an on-line computer the shape of each peak can be examined and assessed as to the likelihood of its being a single component. If more than one component is suspected then the simplex can be restarted in a different region of the factor space: this approach is the subject of current studies.

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