Optimization of separations in supercritical fluid chromatography using a modified simplex algorithm and short capillary columns

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Optimization of Separations in Supercritical Fluid Chromatography Using a Modified Simplex Algorithm and Short Capillary Columns

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A modified simplex algorithm has been used to optimize supercritical fluid chromatography (SFC) separations of samples containing nonhomologous, nonoligomeric components. Short capillary columns are employed in the initial separations, with a transfer to a longer column for greater efficiency as needed. Two chromatographic response functions, based on peak–valley ratio or threshold resolution criteria, were found to be suitable for SFC. Two- and three-variable simplexes utilizing (i) density gradient rate and temperature, (ii) simultaneous pressure and temperature gradient rates, or (iii) initial density, density gradient rate, and temperature provided good results for the samples in this study. Convergence to the global optimum was shown for case i by restarting the simplex in another part of the parameter space. A synthetic mixture of three difficult-to-separate sesquiterpene lactones was separated by optimization on a short column using the three-parameter simplex and then transferring the method to a longer column.

INTRODUCTION

More often than not, the initial separation of a given sample is unsatisfactory, usually because the desired resolution between all the peaks of interest is insufficient. To improve the separation in an efficient manner, an optimization procedure with well-defined goals is strongly recommended (*1*). The goals set may vary depending on how many peaks are of interest, the resolution required, the importance of analysis time, and other considerations. The point at which an optimization procedure is terminated depends on the quality of the separation desired; there is a distinct difference between an acceptable and an optimum separation. The decision upon which optimization is usually based takes into account a minimum resolution in some maximum time frame (*2*). Many chromatographic response functions (CRF's) have been developed and used based on this idea (*3, 4*).

Each of the three terms in the fundamental resolution equation (*5*) can be optimized to improve the separation. Retention (k') and the selectivity (α) should first be optimized via changes in the density of the mobile phase, the temperature, and the gradient rates. Optimization of these two parameters is clearly the first step, since it will indicate if the current mobile phase/stationary phase combination is adequate for the separation being considered. The efficiency of a column, N, is determined by its length and the nature of the stationary phase, including column diameter or particle size. Given the square root dependency of resolution on N, large changes in parameters controlling N (e.g., column length or linear velocity) will result in only moderate changes in resolution and thus should only be considered if changes in the parameters controlling selectivity and retention in SFC (composition, density, temperature (or their respective gradients, if employed)) do not suffice.

Supercritical fluid chromatography (SFC) displays GC- and/or LC-like behavior, depending on both the solutes and the experimental conditions. Some components may partition by their vapor pressures while others partition by solventlike properties of the mobile phase (*6*). As the experimental conditions are changed, the behavior of some or all of these components may be reversed. Elution order may also depend on such properties as basicity and steric hindrance (*7*). Finally, many of the parameters that control retention and selectivity are moderately to highly synergistic (*8*). For these reasons, univariate optimization strategies (sequential optimization of one parameter at a time) or intuitive approaches are often ineffective in locating a true optimum (*3*), hence the need for a simultaneous multivariate approach in order to obtain the best possible separation.

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Historically, SFC has largely been used for the separation of homologous or oligomeric series of compounds (particularly those without chromophores), and methods for these types of separations are relatively easy to develop intuitively. However, as SFC is applied more frequently to samples with diverse, nonhomologous components, it is clear that a better optimization strategy (or theory) will be necessary.

The sequential simplex method is a multivariate optimization procedure that uses a geometrical figure called a simplex to move throughout the response surface in search of the optimum set of experimental conditions (*9*). The simplex has been successfully used in various forms of chromatography, particularly high-performance liquid chromatography (*10–12*) and gas chromatography (*13–16*). To our knowledge, however, the present study represents the first application of the simplex method to SFC. In the present work we investigate the ability of a simplex algorithm to optimize SFC separations. First, the simplex method is used with a synthetic test mixture for initial assessment of the procedure, and then it is applied to some difficult-to-separate sesquiterpene lactones using a more rigorous three-parameter optimization.

THEORY

Simplex Algorithm. In the simplex method, the number of initial experiments conducted is one more than the number of parameters (temperature, gradient rate, etc.) to be simultaneously optimized. These initial experiments establish the vertices of a geometric figure (simplex), which will subsequently move through the parameter space in search of the optimum. Once the initial simplex is established, the vertex with the lowest value is rejected, and a new point is found by reflecting the simplex in the direction away from the rejected vertex. In this way the simplex proceeds toward the optimum set of conditions. Details on the simplex algorithm are available elsewhere (*3*, *4*, *9*, *17–19*).

Some advantages of the simplex method include the following: (1) little chromatographic insight is required, (2) computational requirements (relative to other statistical strategies) are minimal, and (3) any number of parameters may be considered. Some disadvantages of the simplex algorithm are (1) a large number of experiments may be required to find an optimum, (2) little insight into the response surface is provided, and (3) a local rather than a global optimum may be found (*3*). With respect to the latter deficiency, the chances of finding a global optimum are enhanced by using a modified simplex which allows other operations besides reflection, such as expansions and contractions. The chances of a mistaking a local optimum for the global optimum are also reduced by restarting the simplex in a different region of the parameter space. If the same optimum is found after restarting the simplex, it is probable that the global optimum has been found.

Response Functions. For chromatographic optimization, it is necessary to assign each chromatogram a numerical value, based on its quality, which can be used as a response for the simplex algorithm. Chromatographic response functions (CRFs), used for this purpose, have been the topic of many books and articles, and there is a wide variety of such CRFs available (*3*, *4*, *20*, *21*). The criteria employed by CRFs are typically functions of peak–valley ratio, fractional peak overlap, separation factor, or resolution. After an extensive (but not exhaustive) survey, we identified two CRFs that are straightforward and easy to use. We intentionally avoided the more complicated CRFs that include factors of maximum analysis time, minimum retention time, or other arbitrary weighting factors. As discussed by Schoenmakers (*3*), these complex CRFs are neither as versatile nor as desirable as previously believed. The "multiple" weighting factors of these CRFs can usually be reduced to a single weighting factor simply by rearrangement of the CRF.

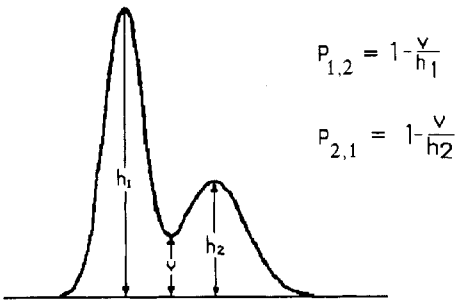

Figure 1. Illustration of the peak–valley ratio measurement used for the optimization process. See eq 2 for the response function used in conjunction with this criterion.

The first CRF we considered uses a threshold criterion based on resolution between peaks (R_s), given the equation

$$\begin{aligned} \text{CRF-1} &= 1/k_\omega \qquad R_{s,\min} \geq x \\ &= 0 \qquad R_{s,\min} < x \end{aligned} \tag{1}$$

In eq 1, k_ω is the capacity factor for the last peak (retention time may be used instead), and $R_{s,\min}$ is the minimum acceptable resolution set arbitrarily by the user. CRF-1 favors chromatograms with a resolution greater than an arbitrary value "x" for all peaks in the shortest amount of time possible. For chromatograms where $R_{s,\min} < x$ for any pair of peaks, the response is set to zero. If the resolution between all pairs of peaks is greater than x, the response is set equal to $1/k_\omega$. Thus as analysis time decreases, the response function value increases provided that the resolution does not fall below the threshold value. For our analyses, $R_{s,\min}$ was chosen to be unity. A different value may be more appropriate in some instances.

An inherent problem with CRF-1 is its inability to distinguish between chromatograms with a resolution below the threshold. All such chromatograms would have a value of zero, among which the algorithm could not differentiate. A more continuous CRF may therefore be desirable in some instances.

The second CRF we considered is a continuous one based on the ratio of peak height to valley depth. There are several ways in which this ratio can be implemented, and the specific method we used, first introduced by Christophe (*22*), is illustrated in Figure 1. The resulting CRF is

$$\text{CRF-2} = \frac{\Pi(P_{i,i-1}P_{i,i+1})^{1/2}}{t_\omega} \tag{2}$$

where, for the ith peak, $P_{i,i-1} = 1 - (v_{i-1}/h_i)$ and $P_{i,i+1} = 1 - (v_{i+1}/h_i)$ (see Figure 1).

CRF-2 also favors short analysis times and well-resolved peaks. There is no threshold value for resolution, and the compromise between resolution and analysis time is not as well-defined as in CRF-1. Inclusion of analysis time in the denominator of an objective function may result in the loss of some information, compensated for by a rapid analysis time (*23*). It is important to note, however, that as peaks become overlapped CRF-2 decreases rapidly, as shown in Figure 2, and it is unlikely that a short analysis time will compensate for poor resolution (*3*). This is true only to a point, however, as the peak–valley ratio utilized by CRF-2 does not diminish to an appreciable extent until the resolution falls below a value of 1 to 1.25. If a minimum resolution is an absolute requirement, it is probably better to use a threshold criterion such as that introduced earlier so that the desired resolution is set by the user.

Solvent Peak. A common problem in SFC is the separation of the solvent peak and the first peak of interest. For reproducibility and quantitation, it is important to separate the peaks of interest from the highly asymmetric, tailing

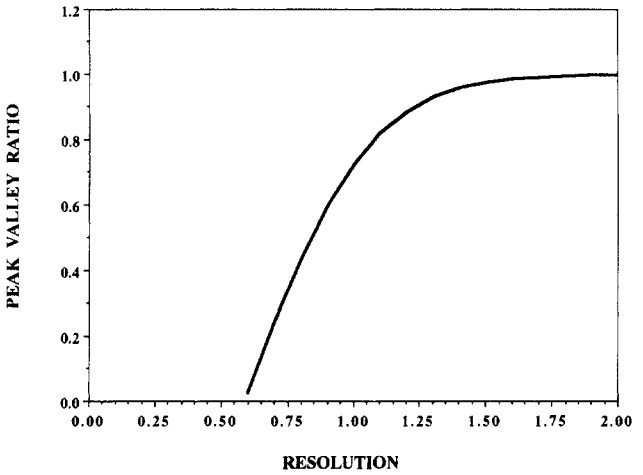

Figure 2. Relationship between resolution and peak–valley ratio as calculated from the equation $P = 1 - 2\exp(-2R_s^2)$. See ref 3 for details.

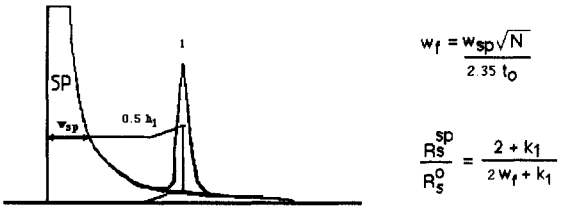

Figure 3. Illustration of the method for determining resolution between the solvent peak and the first peak of interest. The method was taken and modified from that introduced by Schoenmakers et al. (*24*). See eqs 3 and 4 in text.

solvent peak. If CRF-1 or any other CRF that uses resolution (R_s) is employed, a procedure suggested by Schoenmakers et al. (*24*) and modified in our laboratory can be used to measure the resolution between the solvent peak and the first solute peak.

Schoenmakers' calculation uses the width of each peak measured at 13.5% *relative to the solute peak* and a weighting factor proportional to the width of the solvent peak. Our modification is the measurement of both peak widths at 50% relative to the solute peak instead of at 13.5%. We have found the former measurement significantly easier because it more readily avoid problems caused by (i) potential base-line disturbances on the lower part of the solvent peak tail resulting from the start of a gradient or (ii) imprecise peak width measurements resulting from low signal-to-noise ratios (i.e., near the limit of detection).

In order to be equivalent to Schoenmakers' original expression, our modification requires that the coefficient in the denominator of the weighting factor expression be reduced from 4 to 2.35. Our modifications are shown in eq 3 and in Figure 3. In eq 3, w_{sp} is the width of the solvent peak

$$w_f = \frac{w_{sp}N^{1/2}}{2.35t_0} \tag{3}$$

measured at 50% relative to the solute peak, N is the plate count of the column, and t_0 is the retention time of the solvent peak. The solute peak used to calculate the efficiency will obviously affect the value of w_f, and should typically have a k' value between two and four. Note that if the solvent peak was ideal (symmetric instead of tailed), w_f would be unity. For broader solvent peaks, w_f becomes larger. The resolution between the solvent peak and first analyte peak (R_s^{sp}) is calculated by

$$R_s^{sp} = \frac{2 + k_1}{2w_f + k_1}R_s^\circ \tag{4}$$

where $R_s^\circ = \Delta t_R / W_{solute}$ and k_1 is the capacity factor for the solute peak.

Note that no such modifications for the solvent peak are generally necessary for CRFs that employ a peak–valley ratio, since the overlap is measured directly and no assumptions are made concerning peak shape. As the first solute peak becomes more overlapped with the solvent tail, the peak–valley ratio will rapidly decrease toward zero and give rise to an unfavorable response.

Increasing *N*. For a new sample, the time required for analysis must include the development of the method for separation. In the case of SFC, a great amount of time can be saved by performing the method development with a very short column (*25*). This can be done efficiently in combination with the modified simplex algorithm described above. Additional time will of course be saved if this short column proves to be sufficient, once the conditions are optimized, for the final analysis. If the current column does not provide the required efficiency, the resolution can be increased by a factor y via a y^2 increase in column length. If a gradient is being used, the gradient rate should be decreased appropriately. Assuming that ln k vs the variable of interest (density, pressure, or temperature) is linear, the gradient rate should be decreased by y^2 (*26*). Note that although an increase in column length results in a proportional increase in analysis time, hours of analysis time have already been saved by first optimizing the separation on a short column.

If the separation is still unsuitable after optimization of the experimental conditions and column length, selectivity must be optimized further by changing the stationary phase, the type of column, or the mobile phase by changing it or adding a modifier.

EXPERIMENTAL SECTION

SFC System. The chromatographic system consisted of a Model 501 supercritical fluid chromatograph (Lee Scientific, Salt Lake City, UT) with the flame ionization detector (FID) set at 375 °C. The instrument was controlled with a Zenith AT computer. A pneumatically driven injector with a 200-nL or a 500-nL loop was used in conjunction with a splitter. Split ratios used were between 5:1 and 50:1 depending on sample concentration and the chosen linear velocity, while the timed injection duration ranged from 50 ms to 1 s. We found that variation of both the split ratio and the injection time allowed greater control over the amount of solute transferred onto the column. Data were collected with an IBM-AT computer using Omega-2 software (Perkin-Elmer, Norwalk, CT). The simplex program and the response function calculation programs were written in TrueBASIC (TrueBASIC, Inc., Hanover, NH). The capillary columns used were a 0.55-m, a 1-m, and a 3-m SB-Biphenyl-30 (30% biphenyl, 70% methyl polysiloxane), with a 50-μm internal diameter and a film thickness of 0.25 μm. The mobile phase was SFC grade carbon dioxide (Scott Specialty Gases, Baton Rouge, LA). Linear velocities were 1.5 and 2.0 cm/s through the 50-μm frit restrictor, estimated from the retention time of methane at 100 atm and 100 °C. To prevent plugging, the 15-μm split restrictor was run out of the oven into a vial of methylene chloride. This was important in the analysis of the more polar sesquiterpene lactone sample (vida infra). Density was held constant until the solvent had eluted, at which point a gradient program was initiated.

Samples. Two samples were used to test the simplex method. Sample 1 was a synthetic test mixture consisting of six low-to-medium molecular weight solutes (acetophenone, propiophenone, bicyclohexyl, biphenyl, undecylbenzene, and benzophenone) of varying polarity and functionality dissolved in HPLC grade hexane. An injection duration of 100 ms was used with this sample. Sample 2 was a synthetic mixture of three sesquiterpene lactones (glaucolide A, burrodin, and psilostachyin A) dissolved in HPLC grade methylene chloride. An injection duration of 200 ms was used in conjunction with this sample.

Simplex Algorithm. The modified simplex algorithm was based on that used by Nelder and Mead (*27*) except that any vertex obtained through a contraction that had the worst response

Table I. Results of Two-Parameter Simplex, Run 1

vertex	density rate, (g/mL)/min	temp, °C	response (eq 2)	t_w,[a] min	simplex movement	retained vertices
1	0.075	75	0.150	6.51	–	–
2	0.123	88	0.186	5.00	–	–
3	0.088	123	0.122	4.52	–	–
4	0.110	40	$-\infty$[b]	–	reflection	1,2
5	0.094	102	0.175	4.84	c_w contraction	1,2
6	0.142	115	0.187	3.93	reflection	2,5
7	0.175	135	0.0601	3.45	expansion	2,5
8	0.172	101	0.205	3.85	reflection	2,6
9	0.211	100	0.205	3.67	expansion	2,6
10	0.229	128	$-\infty$	–	reflection	6,9
11	0.150	98	0.199	4.24	c_w contraction	6,9
12	0.218	83	0.201	3.94	reflection	9,11
13	0.279	85	$-\infty$	–	reflection	9,12
14	0.182	95	0.221	3.99	c_w contraction	9,12
15	0.174	112	0.193	3.68	reflection	9,14
16	0.207	90	0.234	3.69	c_w contraction	9,14
17	0.179	84	0.227	4.11	reflection	14,17
18	0.204	80	0.227	3.97	reflection	14,17

[a] t_w is the retention time of the last eluting peak. [b] This value is given to conditions that are outside the boundary limits.

was kept and the next-to-worse vertex was rejected instead. This avoids massive contractions which often reduces the parameter space too quickly (see ref 9). Experimental conditions for the initial vertex are chosen intuitively by the user; the remaining vertices of the initial simplex are calculated by the algorithm using a user-specified step size. Boundary conditions for the parameter space were specified according to instrumental limitations or arbitrarily, but with rational user judgement. When the simplex algorithm moved outside the set boundaries, a response value of negative infinity was given to that coordinate. Peak and valley heights were measured from the chromatograms on screen using the data collection system and a program was written to calculate the peak–valley ratio response function from these values. The response function value was subsequently used in the simplex program.

RESULTS AND DISCUSSION

Two-Variable Simplex. While the simplex algorithm can be performed with any number or kind of parameters, the number of experiments required for convergence to an optimum rapidly increases as more parameters are considered (*3*). Also, since the number of parameters changing is larger, the response surface is more complex, i.e., with more local optima, and thus a global optimum may be more difficult to find. For these reasons it is advisable to limit the parameters to the ones believed to be important in the optimization process. For SFC, these include density (or pressure), temperature, mobile phase composition, and gradients of each. To reiterate, the key to a rapid optimization for a given separation is to consider only the most important variables.

In our first attempts at SFC optimization we considered only two parameters: density gradient and column temperature. The boundary limits for this simplex were 0.01–0.4 (g/mL)/min for the density gradient rate and 40–200 °C oven temperature. The linear velocity for this simplex was approximately 1.5 cm/s. Sample 1 described earlier was used to test the optimization procedure. Table I gives the experimental conditions and value of the response function (CRF-2) at each vertex of the simplex. The amount of peak overlap can be ascertained by multiplying the response in the tables by t_w (see eq 2). Also note that the "retained vertices" column refers to the vertices kept just prior to the generation of the current vertex. Figure 4 illustrates the movement of the simplex algorithm and the evolution of the CRF. When the simplex algorithm chose conditions outside the boundary limits, a very negative response value was given to that coordinate. This can be seen in the tables and in the response progress figures where negative columns indicate this very

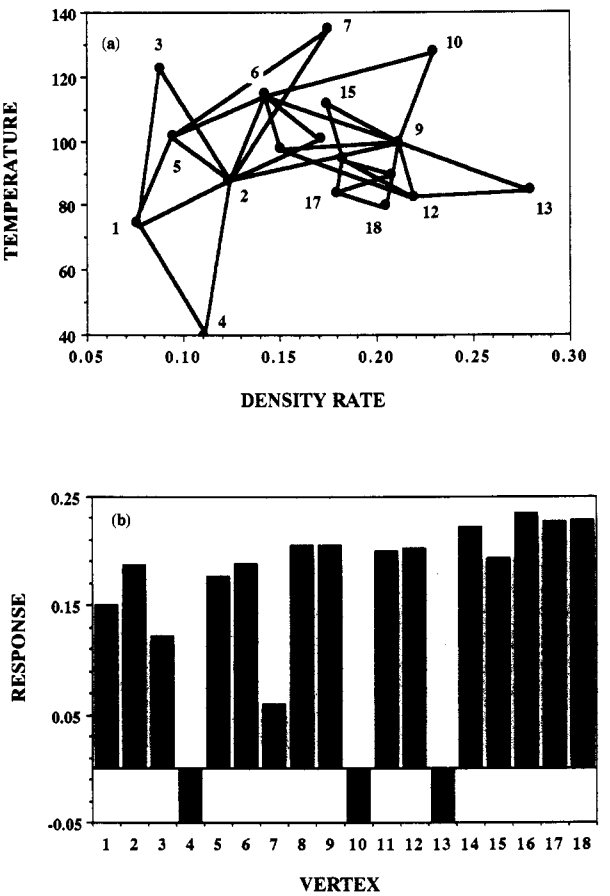

Figure 4. First simplex optimization of density gradient rate and column temperature performed on the test mixture, showing (a) simplex movement and (b) response progress.

negative response. Chromatograms for selected vertices in the simplex are shown in Figure 5. The simplex was terminated after a good separation was obtained under 4 min. The best result was obtained at vertex 16, at a density gradient of 0.207 (g/mL)/min and an oven temperature of 90 °C, as seen in Figure 5d.

Given the possibility of finding a local rather than a global optimum, the simplex was restarted from another region of the parameter space by using the same boundary limits and linear velocity as before. The data for this second simplex are given in Table II. Figure 6 shows the simplex movement

Table II. Results of Two-Parameter Simplex, Run 2

vertex	density rate, (g/mL)/min	temp, °C	response (eq 2)	t_w,[a] min	simplex movement	retained vertices
1	0.150	50	0	–	–	–
2	0.247	63	0.103	4.36	–	–
3	0.176	98	0.215	4.26	–	–
4	0.272	111	$-\infty$[b]	–	reflection	2,3
5	0.181	65	0.171	5.17	c_w contraction	2,3
6	0.110	101	0.164	5.02	reflection	3,5
7	0.144	91	0.202	4.10	c_r contraction	3,5
8	0.139	124	0.122	3.75	reflection	3,7
9	0.170	80	0.201	4.50	c_w contraction	3,7
10	0.202	87	0.236	3.80	reflection	3,9
11	0.231	85	0.223	3.93	expansion	3,9
12	0.237	103	0.211	3.53	reflection	3,11
13	0.220	97	0.239	3.49	c_r contraction	3,11
14	0.275	84	$-\infty$	–	reflection	11,13
15	0.201	95	0.123	3.87	c_w contraction	11,13

[a] t_w is the retention time of the last eluting peak. [b] This value is given to conditions that are outside the boundary limits.

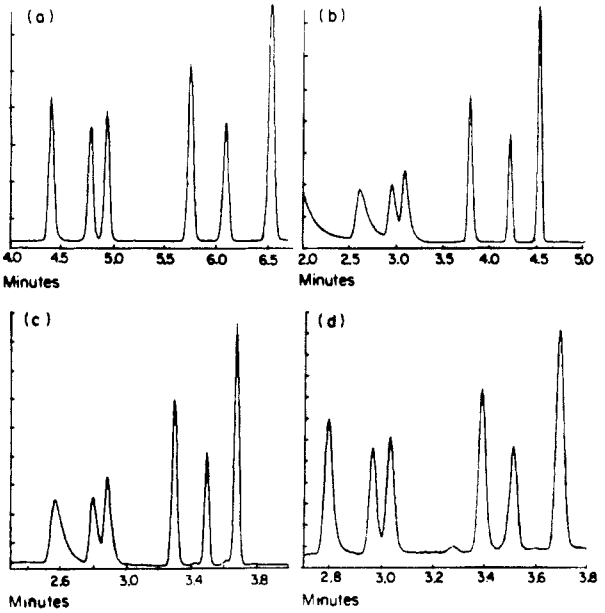

Figure 5. Four chromatograms from the first two-parameter simplex: (a) vertex 1, (b) vertex 3, (c) vertex 15, (d) vertex 16. Components are, from left to right, hexane (solvent), acetophenone, propiophenone, bicyclohexyl, biphenyl, undecylbenzene, and benzophenone. See Table I for conditions and response values for these chromatograms. Signals corresponding to tallest peaks were (a) 26, (b) 44, (c) 48, and (d) 17.2 pA.

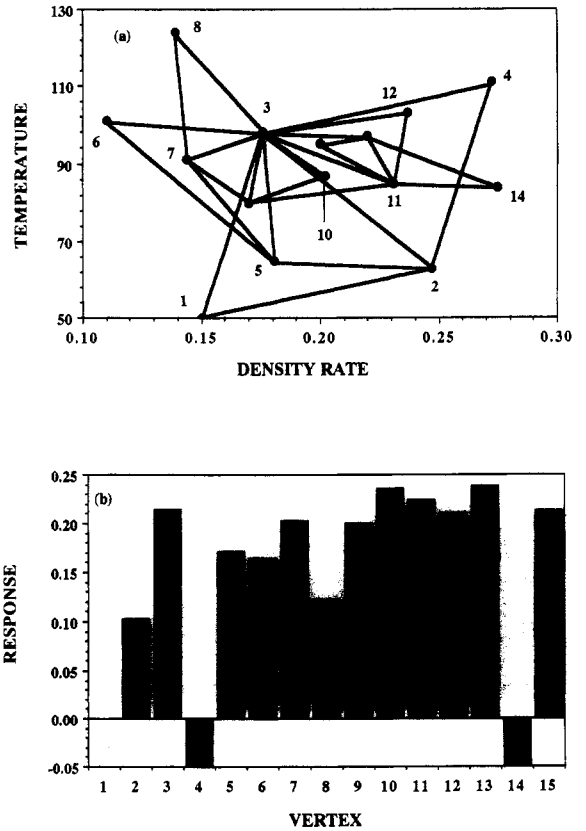

Figure 6. Second run for the simplex optimization of density gradient rate and column temperature performed on the test mixture, showing (a) simplex progress and (b) response progress.

and the progression of the CRF values. The best response was obtained at vertex 13, with a density gradient of 0.22 (g/mL)/min and an oven temperature of 97 °C. The response at vertex 13 (0.239) is essentially equivalent to that at vertex 16 (0.234) in the first simplex (cf. Tables I and II). The somewhat faster convergence of the second simplex is explained by its larger initial step sizes, thus requiring less time to reach the optimum region. Nonetheless, since both trial runs converged to essentially the same conditions, it is highly probable that the global optimum has been reached.

From Tables I and II it can be seen that the last vertices in the simplex are not always the ones with the best response. This is due to the fact that once an optimum region is reached, the simplex begins to "circle" the optimum. At this point it is advisable to discontinue the simplex, as many experiments could be wasted in the close vicinity of the optimum (*3*). In our view, whenever a set of experimental conditions that provides the desired separation within the maximum specified analysis time has been found, the optimization procedure (method devlopment) can be halted. Although we feel that this is the most practical criterion for ending a simplex, other more rigorous criteria for simplex termination are also available. One such criterion is based on a comparison of the relative change in the various experimental conditions (*17*). For the last three vertices of the first simplex (Table I), the relative standard deviation for the density rate and temperature are 8% and 6%, respectively. Although somewhat higher than desirable, we believe these data indicate that the predicted optimum for density rate and temperature are close to the true optimum.

Whereas a linear density gradient at constant temperature may result in a more predictable solvent strength program, asymptotic density gradients have been shown to give a better separation for the later-eluting oligomeric peaks of higher molecular weight samples (*28*), particularly when a temperature gradient is performed simultaneously (*29*). Unfortu-

Table III. Results of Two-Parameter Simplex, Simultaneous Gradients

vertex	pressure rate, atm/min	temp rate, °C/min	response (eq 2)	t_w,[a] min	simplex movement	retained vertices
1	30	20	0.263	3.61	–	–
2	69	25	0.326	2.62	–	–
3	40	39	0.283	3.15	–	–
4	79	44	$-\infty$[b]	–	reflection	2,3
5	42	26	0.283	3.23	c_w contraction	2,3
6	71	12	0.332	2.59	reflection	2,5
7	86	-1.7	$-\infty$	–	expansion	2,5
8	97	11	0.296	2.33	reflection	2,6
9	83	15	0.341	2.47	c_r contraction	2,6
10	85	1.6	$-\infty$	–	reflection	6,9
11	73	19	0.333	2.59	c_w contraction	6,9
12	85	22	0.342	2.47	reflection	9,11
13	93	27	0.345	2.38	expansion	9,11
14	103	23	0.325	2.31	reflection	9,13
15	80	20	0.344	2.51	c_w contraction	9,13
16	90	33	0.338	2.42	reflection	13,15
17	85	19	0.331	2.46	c_w contraction	13,15
18	98	26	0.338	2.36	reflection	13,17

[a] t_w is the retention time of the last eluting peak. [b] This value is given to conditions that are outside the boundary limits.

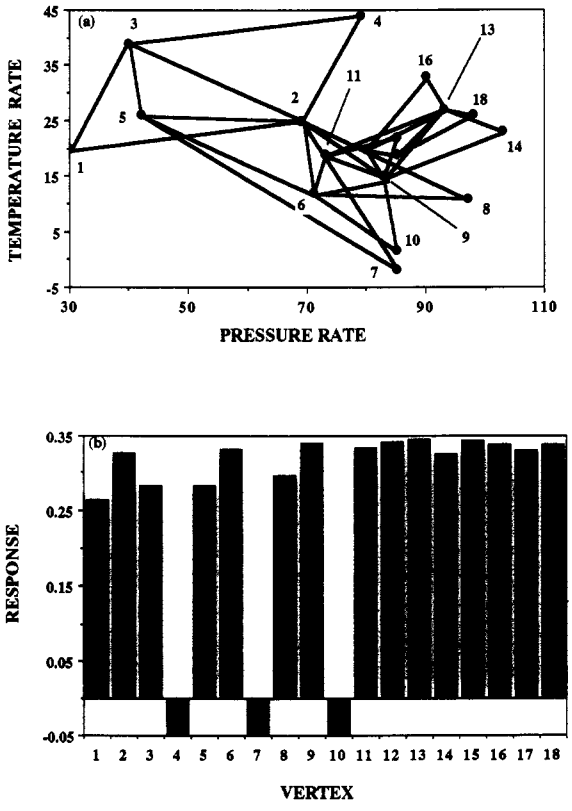

Figure 7. Simplex optimization using two simultaneous gradients (pressure and temperature) performed on the test mixture, showing (a) simplex progress and (b) response progress.

nately, asymptotic gradients are more tedious to generate experimentally, and this disadvantage would be exacerbated during the course of a simplex run. Fortunately, however, asymptotic density gradients can usually be approximated by a linear pressure program. Thus in order to consider the possible benefits of optimizing these variables, a third simplex algorithm was run by using simultaneous linear pressure and temperature gradients on this same test mixture used in simplex runs 1 and 2. The boundary limits for this simplex were 5–150 atm/min and 5–40 °C/min. The data for this simplex are given in Table III, Figure 7a shows the simplex progress, and the CRF evolution is shown in Figure 7b. Two chromatograms for this optimization are shown in Figure 8.

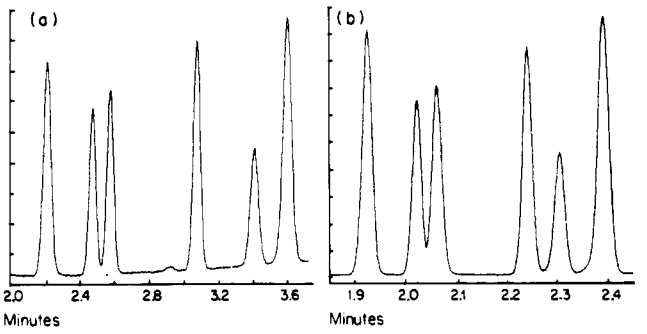

Figure 8. Two chromatograms from the simultaneous gradient simplex: (a) vertex 1 and (b) vertex 13 (best response). See Table I for conditions and response values for these two chromatograms. Signals corresponding to tallest peaks were (a) 22 and (b) 52 pA.

Figure 8a is the chromatogram obtained for the initial vertex, while Figure 8b is the chromatogram for vertex 13, at which the best response was obtained. The major improvement observed is about a 33% reduction in analysis time. For separations to be transferred to a longer column, this reduction of analysis time becomes very significant. For this separation, a higher linear velocity of 2 cm/s was used, resulting in a shorter analysis time than that observed in Figure 5, and thus higher response function values. The temperature gradient reduced peak tailing (cf. Figure 5 and 8) and allowed closer peak spacing without overlap, also contributing to the shorter analysis time.

Three-Variable Simplex. While the above optimizations provide a useful graphical representation of the simplex progress, the initial density (or pressure) is also an important parameter that should be considered in SFC optimizations. We have found that, other factors being equal, the starting density determines the highest initial temperature that can be used without merging the first solute peak and the solvent peak. For this reason the starting density was also included as a parameter in the simplex.

This three-variable simplex algorithm was employed to optimize the separation of a sesquiterpene lactone sample. One of the largest groups of plant products, sesquiterpene lactones possess great biological activity and are useful in many medical and agricultural capacities (*30*). Separation techniques often used for these compounds include column liquid chromatography, gas chromatography (GC), and reversed-phase high-performance liquid chromatography (HPLC).

Table IV. Results of Three-Parameter Simplex

vertex	initial density, g/mL	density rate, (g/mL)/min	temp, °C	response (eq 2)	t_w,[a] min	simplex movement	retained vertices
1	0.250	0.100	60	0.132	4.74	-	-
2	0.486	0.171	84	0	-	-	-
3	0.309	0.383	84	$-\infty$[b]	-	-	-
4	0.309	0.171	154	0	-	-	-
5	0.387	-0.089	115	$-\infty$	-	reflection	1,2,4
6	0.329	0.265	91	0.0901	2.09	c_w contraction	1,2,4
7	0.401	0.186	2	$-\infty$	4.74	reflection	1,2,6
8	0.332	0.175	116	0	1.73	c_w contraction	1,2,6
9	0.121	0.189	95	$-\infty$	-	reflection	1,6,8
10	0.395	0.175	86	0.157	2.06	c_w contraction	1,6,8
11	0.317	0.186	42	$-\infty$	-	reflection	1,6,10
12	0.328	0.177	98	0.055	2.41	c_w contraction	1,6,10
13	0.320	0.037	71	0.129	4.66	reflection	1,10,12
14	0.315	0.031	47	$-\infty$	-	reflection	1,10,13
15	0.325	0.141	85	0.131	2.73	c_w contraction	1,10,13
16	0.326	0.241	83	0.128	2.43	reflection	1,10,15
17	0.322	0.088	74	0.146	3.52	c_w contraction	1,10,15
18	0.319	0.101	62	0.163	3.76	reflection	1,10,17
19	0.316	0.082	50	0	-	expansion	1,10,17
20	0.440	0.143	88	0	-	reflection	10,17,18
21	0.298	0.111	67	0.132	3.73	c_w contraction	10,17,18
22	0.231	0.025	49	$-\infty$	2.73	reflection	17,18,21
23	0.354	0.138	77	0.136	2.85	c_w contraction	17,18,21
24	0.365	0.107	75	0.141	2.47	reflection	17,18,23
25	0.317	0.060	64	0.143	2.38	reflection	17,18,24
26	0.273	0.059	58	0.128	2.31	reflection	17,18,25
27	0.342	0.095	71	0.131	2.51	c_w contraction	17,18,25
28	0.338	0.130	74	0.147	2.42	reflection	17,18,27
29	0.310	0.117	69	0.130	2.46	reflection	17,18,28
30	0.334	0.101	70	0.133	2.36	c_w contraction	17,18,28

[a] t_w is the retention time of the last eluting peak. [b] This value is given to conditions that are outside the boundary limits.

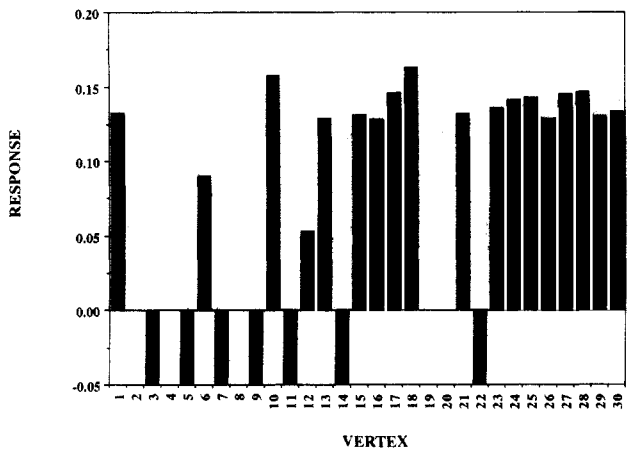

Figure 9. Response progress for the three-parameter simplex on the sesquiterpene lactone sample.

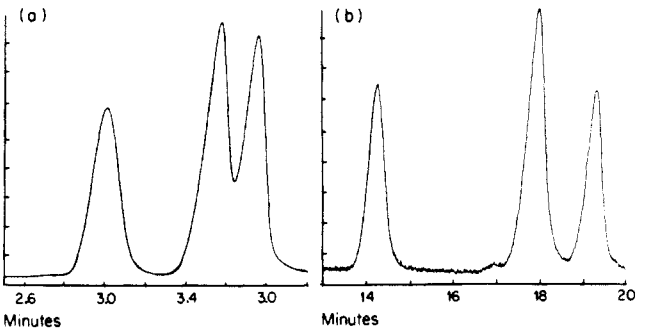

Figure 10. Chromatograms from the sesquiterpene lactone sample optimization for (a) the separation with the best response from the simplex (vertex 18) and (b) the separation after transfer to the 3-m column. Conditions for chromatogram a are given in Table III and for chromatogram b were 0.319 g/mL initial density, 0.01 g/(mL/min) gradient, and 62 °C oven temperature. Components are, from left to right, psilostachyin A, burrodin, and glaucolide A. Signals corresponding to tallest peaks were (a) 44 and (b) 16 pA.

None of these methods, however, has proven to be completely satisfactory. Classical column chromatography does not always provide the needed resolution, GC analysis may result in thermal degradation of the sample, and HPLC is limited by the lack of a sensitive universal detector.

For these reasons, SFC would appear to be a promising alternative for the separation of these compounds. To our knowledge, the present study represents the first separation of this class of compounds by SFC. Although somewhat polar and perhaps more amenable to a modified CO_2 mobile phase, at least some sesquiterpene lactones can be separated with pure CO_2 (vide infra).

For the optimization of the sesquiterpene sample, a 55-cm SB-Biphenyl-30 column with an internal diameter and film thickness of 50 and 0.25 μm, respectively, was used for the separation. Boundary limits were 0.2–0.5 g/mL (initial density), 0.01–0.4 (g/mL)/min (density gradient rate), and 50–200 °C (temperature). Table IV gives the experimental parameters used in the simplex and the responses for the vertices; simplex movement was not shown due to the difficulty of graphically representation of a three-dimensional figure in two dimensions. Figure 9 shows how the CRF evolved as the simplex progressed. After 30 vertices the simplex was terminated, with vertex 18 giving the best response with initial density of 0.319 g/mL, a 0.101 (g/mL)/min density gradient after solvent elution, and an oven temperature of 62 °C. Figure 10a shows the optimized chromatogram for this short column.

Increasing *N* for the Final Separation. We have found that it is worthwhile to have several different lengths of columns on hand so that the efficiency can be varied as necessary once the selectivity and retention have been op-

timized. From Figure 10a it is clear that the 55-cm column does not provide sufficient resolution for this sesquiterpene lactone sample. The resolution in this chromatogram is about 0.4 for the last two peaks. Transferring the method to a 300-cm column, an increase in length of 5.5, should increase R_s by a factor of $5.5^{1/2}$ to a value near unity. The corresponding gradient rate to use would be (0.101 (g/mL)/min)/5.5 = 0.018 (g/mL)/min. To achieve a resolution greater than unity, we employed a slightly lower gradient rate of 0.010 (g/mL)/min. Note that we intuitively selected this lower gradient rate for the final separation to provide a slightly higher resolution. The resulting separation is shown in Figure 10b. A decrease in the signal to noise ratio is apparent here, resulting from longer analysis times which decrease the peak heights.

Response Functions. The continuous CRF used above (CRF-2) proved to be the most efficient and successful of all the CRFs we examined. Calculation of CRF values was quick and straightforward, aided by a simple, yet effective, computer program. As can be seen from Figure 5, however, and as stated in the theory section, continuous CRFs do compromise resolution with analysis time *up to a point*. If a *threshold* CRF with $R_{s,min} = 1.5$ (e.g., CRF-1) been used in the first simplex instead of the *continuous* CRF-2, vertex 16 would not have been the best set of conditions but would rather have received a value of zero because of peaks two and three (see Figure 5). The simplex would have obviously taken a different course dictated by that criterion.

If column efficiency is constant throughout an optimization procedure, a useful parameter to use in eq 1 instead of R_s would be the separation factor S, defined as (*31*)

$$S = \frac{t_2 - t_1}{t_1 + t_2} \tag{5}$$

Equation 5 has the advantage of easily being obtained from the chromatogram and is related to resolution in the following manner:

$$R_s = S^* \frac{N^{1/2}}{2} \tag{6}$$

Unfortunately we cannot always assume that the column efficiency will be constant for all peaks in a chromatogram, or for chromatograms run under different conditions. As shown by Snyder, et al. (*32*), the bandwidth of a peak observed in gradient elution is reduced compared to that obtained under nongradient conditions. The factor by which the bandwidth is reduced is usually a function of gradient steepness and is also determined by the instantaneous value of k' as the solute leaves the column. This reduction in bandwidth cannot be estimated under nongradient conditions, so that in order to correctly use the separation factor approach, bandwidths for each chromatogram under different gradient conditions would have to be calculated. In general, it is easier to measure resolution directly using eq 7

$$R_s = \frac{t_2 - t_1}{w_{avg}} \tag{7}$$

where t_1 and t_2 are the retention times and w_{avg} is the average width of the peaks at the base line. For pairs of peaks with resolution obviously greater than that desired, a measurement is not needed for threshold CRFs like CRF-1.

Solvent Peak. The anticipated problem of separating the first solute peak from the solvent peak was solved by using a response function which severely penalizes such overlap. Since gradients were initiated immediately after the solvent had eluted, analyte peaks on or near the tail of the solvent peak were broad because they were eluted under nongradient conditions. Figure 5b demonstrates this effect. Since the peaks eluting under nongradient conditions are not focused, the relative height of the valley is greater and the chromatogram is thus penalized. When the first solute peak elutes late enough to be truly influenced by the gradient, it will receive a better value from the response function. Our results indicate that it requires less than 1 min of gradient conditions for the first solute peak to be sharp and well-separated from the solvent.

Since the length of time necessary to elute the solvent will change depending on initial conditions, it may be difficult to determine the hold time needed. Although it is reasonable to use the longest hold time that will be encountered for any one chromatogram for all the chromatograms considered, doing this will penalize those chromatograms in which the solvent elutes well before the end of the set hold time. A better approach is either to manually start gradients once the solvent has eluted or to run a solvent blank before each sample to determine the hold time. Alternatively, if the linear velocity could be accurately predicted, the elution time of the solvent for a given set of experimental conditions could be predicted, assuming the solvent was more or less unretained ($k' = 0$). A more sophisticated solution would be the use of a data feedback system to start the gradients once the detector signal returns to a given value after solvent elution.

CONCLUSIONS

A systematic method development scheme is clearly desirable for SFC, and as we have shown in the present work, the modified simplex algorithm is a promising approach to the optimization of SFC separations. By use of a short capillary column and first optimizing the selectivity and retention, rapid separations are possible in the development stage, with the potential of optimizing efficiency later if needed. This saves hours of analysis time, especially if the short column proves to be sufficient for the final separation. Confidence that the global optimum has been found is provided by the convergence to the same conditions of two simplexes started at different points within the parameter space.

Since our report represents the first application of the simplex algorithm in SFC, there are many avenues open for development. Although we have demonstrated the potential of simplex optimization in SFC and have shown how it can be successfully implemented, additional studies focusing on the selection of the best combinations of experimental parameters to optimize are clearly warranted. Other opportunities for research include the extension of the simplex method to packed columns and modified mobile phases and a detailed evaluation of other response functions not examined in the present study. Finally, other optimization strategies, such as a grid search (*33*), factorial design (*34*), or window diagrams (*35*) may also prove to be useful.

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Determination of Iodine in Oyster Tissue by Isotope Dilution Laser Resonance Ionization Mass Spectrometry

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The technique of laser resonance ionization mass spectrometry has been combined with isotope dilution analysis to determine iodine in oyster tissue. The long-lived radioisotope, ^{129}I, was used to spike the samples. Samples were equilibrated with the ^{129}I, wet ashed under controlled conditions, and iodine separated by coprecipitation with silver chloride. The analyte was dried as silver ammonium iodide upon a tantalum filament from which iodine was thermally desorbed in the resonance ionization mass spectrometry instrument. A single-color, two-photon resonant plus one-photon ionization scheme was used to form positive iodine ions. Long-lived iodine signals were achieved from 100 ng of iodine. The precision of $^{127}I/^{129}I$ measurement has been evaluated by replicate determinations of the spike, the spike calibration samples, and the oyster tissue samples and was 1.0%. Measurement precision among samples was 1.9% for the spike calibration and 1.4% for the oyster tissue. The concentration of iodine determined in SRM 1566a, Oyster Tissue, was 4.44 μg/g with an estimate of the overall uncertainty for the analysis of ±0.12 μg/g.

Iodine is an essential trace element for man. Its accurate measurement in foods is vital to understanding human dietary intake and verifying that minimum daily allowances are observed, especially in restricted diets such as infant formulations. Isotope dilution mass spectrometry (IDMS) is an inherently accurate technique (*1*), a "definitive method" for which systematic errors can be thoroughly evaluated. In general, the quantity of an element is determined by IDMS by measurement of the change in isotopic ratio that is produced by adding a calibrated amount of an enriched isotope of the element to the sample. As such, the technique can only be used for elements with more than one isotope. Iodine has only one stable isotope in nature, ^{127}I. However, the radioisotope ^{129}I is long-lived (half-life, 1.59×10^7 years) and available. Thus, the accurate measurement of I in biological and botanical matrices by using IDMS is made possible.

Mass spectrometric methods have been developed both to measure isotope ratios of iodine for IDMS and to measure ultratrace amounts of ^{129}I in the environment, which is typically at levels 10^{-6} to 10^{-12} of stable ^{127}I, itself at part-per-million levels in botanical and biological material. A negative thermal ionization (TIMS) technique has been developed by Heumann et al. They have used IDMS to determine I in salt, chemicals, food, and water (*2-4*). A second negative thermal ionization technique has been published by Delmore in which lanthanum hexaboride is cataphoretically deposited onto a rhenium ionization filament. This filament treatment lowers the work function of the rhenium and results in high ionization efficiency and measurement sensitivity for iodine (*5*). Delmore's technique was adapted in our laboratory and used to determine iodine in SRMs 1572 (Citrus Leaves) and 1549 (Powdered Milk) by IDMS (*6*).

Secondary ionization mass spectrometry (SIMS) was initially investigated to measure ^{129}I (*7*). Ion sputtering ionization, as is done in SIMS, is also used in accelerator mass spectrometry (AMS), which has achieved the lowest detection limits for ^{129}I (*8*). AMS effectively eliminates the limiting background of isobaric molecular interferences which are observed in both secondary ionization and thermal ionization mass spectrometry. The detection of ^{129}I in the environment after the Chernobyl reactor accident is an example of AMS capabilities (*9*).

Laser resonance ionization mass spectrometry (RIMS) has been studied in our laboratory for possible application to ^{129}I