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EFFECTS OF DIFFERENT ORGANIC MODIFIERS IN OPTIMIZATION OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC GRADIENT ELUTION OF A MIXTURE OF NATURAL SECOIRIDOID COMPOUNDS

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SUMMARY

Optimization of the linear gradient elution of a crude extract of *Swertia* herb (Gentianaceae) was performed by means of sequential methods (Fibonacci and simplex) using a reversed-phase partition system and three different organic modifiers. The optimum gradient elution separations obtained with methanol, acetonitrile and tetrahydrofuran as different organic modifiers are discussed and compared by using the linear solvent strength theory. The Poisson character of the optimized separations was checked and the Davis-Giddings statistical overlap theory was applied in order to calculate the number of components. The value obtained is independent of both the organic modifier and column type. The quality of the best separation obtained was close to the absolute optimum expected from the Davis–Giddings theory. The usefulness of dealing with two or more optimized gradient separations is discussed. Solvent strengths are reported for selected compounds (amarogentin, amaroswerin and gentiopicroside).

INTRODUCTION

In natural product chemistry, whenever a multi-component mixture has to be analysed, high-performance liquid chromatographic (HPLC) gradient elution is strongly recommended as one of the most versatile analytical procedures[1]. However, setting up the analytical conditions requires an accurate optimization procedure. In fact, often not only individual specific compounds have to be determined, but the complexity of the overall mixture must also be established. An example of a natural mixture that has recently been considered is one containing secoiridoid glycosides,

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which are the most important bitter principles in Gentianaceae plant extracts. Such a multi-component mixture produces mixed sensory signals and it therefore has to be completely and homogeneously resolved in order to establish pertinent taste–chemical property correlations. However, this final goal cannot be completely achieved, even by an optimized HPLC single gradient elution, owing both to the limited peak capacities (N_c; see the list of symbols at the end of the paper) currently available and to the mixture complexity. The peak capacity can be straightforwardly increased by multi-dimensional chromatography[2], and general overall information might be enhanced by applying two or more chromatographic systems, *e.g.*, by changing either the column or the organic modifier. However, in the latter instance, the retention sequence, and hence the resulting peak overlapping pattern, should be different.

In this work, the role of the organic modifier was studied in relation to optimization of gradient elution. A combined approach based on Snyder and co-workers' linear solvent strength theory (LSS)[3,4] with further sequential optimization was applied[1,5]. The qualities of the optimizations achieved by using different organic modifiers were compared and the separations obtained were tested by applying the Davis–Giddings statistical method for estimation of the number of components (m) and extent of separation (γ)[6,7]. This approach has already been set up for gradient elution separations of chamomile extracts with methanol as organic modifier and several partition systems[1,5]. In this study, this strategy was applied to the analysis of *Swertia* herb plant by considering three different organic modifiers: methanol, tetrahydrofuran (THF) and acetonitrile. In this way the role of the optimum searching methods and the usefulness of the LSS theory in multi-component mixture gradient elutions were checked under more varied experimental conditions.

Some work has been done on isocratic elution with regard to *Swertia* herb extract but only a few examples of analysis using gradient elution have been reported[8–12]. No systematic attempt has yet been made to optimize the extent of separation.

PROCEDURE

In order to develop a gradient elution optimization strategy, LSS theory is a useful starting point[3,4]. The gradient programme is set up with a convenient solvent strength value according to the following relationship:

$$\Delta\varphi/\Delta t = b/(St_0) \tag{1}$$

where $\Delta\varphi$ is the difference between the final and the initial volume of the organic solvent, Δt the gradient time, b a suitable gradient steepness constant[5], S the solvent strength and t_0 the retention time of an unretained compound.

As previously described[1], the starting S value is tentatively identified as the common S value of the compounds of interest, amarogentin, amaroswerin and gentiopicroside (Fig. 1). The S values are evaluated as usual through the relationship

$$\log k' = \log k_w - S\varphi \tag{2}$$

where k' is the isocratic capacity factor for a selected volume fraction φ of organic solvent and k_w the extrapolated value of k' for $\varphi = 0$. It was assumed that the

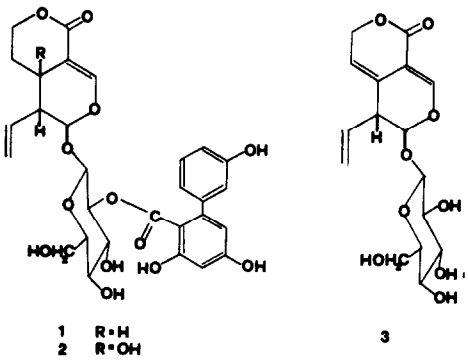

Fig. 1. Secoiridoid glycosides: 1 = amarogentin; 2 = amaroswerin; 3 = gentiopicroside.

chromatographic zones under investigation contained compounds with common solvent strengths, although this might not be the case. However, the results of the subsequent experimental gradient optimization show whether that starting point was correct. The response undergoing optimization was the number of maxima, that is, the number of peaks resolved (p). In this way, a resolution (R_s) of 0.5 between adjacent peaks is assumed[1,6]. In this manner, a single "peak" may include more than one component. However, as different relative concentrations of various components can be present, this statement ($R_s = 0.5$) is only approximate. Nonetheless, this assumption is only related to the procedure for the evaluation of the number of components (see below) and a numerical simulation procedure will be applied in order to guarantee coherence of the results obtained. No attempt has been made here to evaluate peaks at $R_s = 1$ as it was difficult to make an accurate identification of the baseline at the wavelength employed (250 nm). The critical noise level was fixed at 0.003 absorbance; all maxima beneath this level were disregarded so as to avoid counting peaks arising from uncontrolled noise. Consequently, a filter (0.3% of the standard full scale used) was set up to filter out any minor component peaks.

The best gradients obtained with different organic modifiers were compared with each other with reference to several main features. Among these, the standardized analysis time is defined as follows:

$$\tau_{tot} = (t_{tot} - t_0)/t_0 \tag{3}$$

where t_{tot} is the total gradient time calculated according to eqn. 1:

$$t_{tot} = (\varphi_f - \varphi_i)\,(S/b)/t_0 \tag{4}$$

where φ_f and φ_i are the final and initial organic modifier volume fractions, respectively, corresponding to the last and first peak positions in the chromatogram. The standardized analysis time, τ_{tot}, is an adimensional quantity, equal to the maximum capacity factor spanned on the gradient elution chromatogram. By combining the peak capacity, N_c, and the standardized analysis time, τ_{tot} (eqn. 3), the peak capacity per unit capacity factor is calculated as

$$N_{c,k'} = N_c/\tau_{tot} \tag{5}$$

This last quantity may be interpreted as a sort of peak-producing rate of the actual gradient programme.

Once the optimized gradients have been defined, the Davis–Giddings statistical overlap theory[1,6] is applied to evaluate the number of components m according to

$$\ln p = \ln m - m/N_c \tag{6}$$

and the procedure is validated by numerical simulation[1,13–15]. It may be noted that the observed number of components refers to components at concentrations greater than 0.3% of that of the main component giving approximately a full-scale signal on the standard scale used.

The quality of the best separation attained is subsequently evaluated by computing the extent of separation (v) and the quantities α and β[6,7], defined as

$$\gamma = p/m \tag{7}$$
$$\alpha = m/N_c \tag{8}$$
$$\beta = s/N_c \tag{9}$$

where s is the number of singlet peaks at a given resolution, *i.e.*, the number of peaks representing just a single compound. The quantity s can be calculated by using the equation

$$s = m \exp(-2\alpha) \tag{10}$$

derived by Davis and Giddings[6] for chromatographic separations showing a Poisson-like distribution once the value of α has been defined. One may describe v as the effective degree of separation and β as an index of a sort of ratio of "profit" *vs.* "expenses" in the particular separation to be achieved. In fact, the former is the singlet number (s) obtained and the latter is the peak capacity (N_c) set up.

EXPERIMENTAL

Plant material and standard compounds

Commercial *Swertia* herb was extracted with aqueous ethanol at room temperature. A small amount (2.65 g) of the extract was evaporated to dryness under reduced pressure and the residue was dissolved in 13 ml of methanol. The resulting solution was filtered on a Sep-Pak C_{18} cartridge (Millipore, Bedford, MA, U.S.A.). The sample obtained was used for gradient elution chromatography. Amarogentin, amaroswerin and gentiopicroside were previously isolated and characterized in our laboratories according to the method described by Inouye *et al.*[16]. They were pure enough to be considered as standard compounds.

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) Model 600 multi-solvent delivery system equipped with a Rheodyne (Cotati, CA, U.S.A.) injector valve (20-μl sample loop) and a Waters 990 photodiode-array detector coupled with an APC III personal computer (NEC, Tokyo, Japan) were used for gradient elution chromatography and

peak purity check controls. Capacity factors (k') of the standard compounds were evaluated with a Series 10 liquid chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a Rheodyne injection valve (6-μl sample loop), an LC 85B variable-wavelength UV–visible detector (190–600 nm) (Perkin-Elmer) set at 250 nm and a Sigma 15 integrator (Perkin-Elmer).

Three columns were used: a 25 cm × 4.6 mm I.D. 10-μm C_{18} Sil-X-10 column (Perkin-Elmer), a 25 cm × 4.6 mm I.D. 5-μm C_{18} Bakerbond column (J. T. Baker, Phillipsburg, NJ, U.S.A.) and a 30 cm × 4.6 mm I.D. μBondapak 10-μm C_{18} column (Waters).

Reagents and solvents

All solvents and solutes were of HPLC grade (Rudi-Pont, Hetalab Chemical, Parsippany, NJ, U.S.A.) and analytical-reagent grade, respectively. The mobile phase was composed of organic solvent and purified water (Milli-Q; Millipore, Bedford, MA, U.S.A). The aqueous phase was buffered at pH 2.5–3.5 with 80 m*M* acetic acid and 8 m*M* disodium hydrogenphosphate. Solvent mixtures were filtered on a 0.2-μm Millipore filter and degassed with pure helium.

Chromatographic operations

Gradient elution was performed with two solvents: solvent A [aqueous buffer – organic solvent (95:5)] and solvent B [organic solvent – aqueous buffer (95:5)]. The gradient elutions were programmed to run from 100% solvent A to 100% solvent B for CH_3OH and THF and from 100% solvent A to 55% A–45% B for CH_3CN as modifier. No peaks were observed beyond that limit for relative CH_3CN concentration. Capacity factors were measured under isocratic conditions at a flow-rate of 2 ml/min; the retention time t_0 was measured after the injection of an aqueous unretained probe (1% potassium nitrate). Constant b in eqns. 1 and 4 was taken to be equal to 0.2 and 0.1 for 10- and 5-μm columns, respectively[5]. Gradient times were calculated according to eqn. 4. Hence any experimental gradient programme set up for a column can be shifted to another by simply taking the appropriate b value. Moreover, a comparison between retention data obtained with different C_{18} columns must take into account the appropriate column scaling constant.

Calculations

The optimized quantity was S/b. A Fibonacci search[17] was employed with THF and CH_3CN. For CH_3OH a variable-size simplex optimization was performed using the Instrumentune-Up program[18]. The starting fields for the Fibonacci search were 0–200 and 10–145 for CH_3CN and THF, respectively. The Poisson character of the retention time distribution in gradient elution chromatograms was positively confirmed by a chi-squared test as described previously[1].

The peak capacity N_c was calculated as follows: three standard compounds (amarogentin, amaroswerin and gentiopicroside) were injected and eluted by a suitable gradient elution programme. The peak capacities at $R_s = 0.5$ and 1 are expressed as

$$N_c[R_s = 0.5] = 2(x_f - x_i)/x_0 \quad (11)$$

$$N_c[R_s = 1] = (x_f - x_i)/x_0 \quad (12)$$

where x_f and x_i are the last and first peak position, respectively, chosen as limits of the useful chromatographic space, and x_0 is the mean peak width at the baseline[1].

RESULTS AND DISCUSSION

Isocratic measurement of solvent strength

Solvent strength and log k_w values, calculated by using eqn. 2, are reported in Table I. An example of the fitting according to eqn. 2 is shown in Fig. 2. It can be seen that even if the experimental points do not follow perfectly a linear approximation, they define approximately constant slopes. The S values obtained for these compounds agree with those obtained for flavonoid compounds with the same column and mobile phase[6,19,20]. It must be pointed out that a specific solvent selectivity does not exist as far as the hydroxyl group is concerned. In fact, it can be seen that the Δlog k' contribution arising from this group is relatively constant within the solvent composition range studied and is independent of the organic modifier (see Table II and Fig. 2). Nonetheless, with respect to the overall mixture, solvent selectivity can be focused on if the log k' value for amarogentin is compared with that for gentiopicroside where the biphenyl moiety is absent (Table II). This observation might not appear particularly useful as compounds 1 and 3 show substantial structural differences. However, when a complex mixture containing a large number of components is analysed, the use of CH_3CN, rather than CH_3OH or THF, should generate fairly different overlapping patterns on the chromatograms.

The solvent eluting power determined from S values is THF $\approx$ CH_3OH $\ll$ CH_3CN, whereas the power determined by those φ values for which $k' > 1$ is

TABLE I

RETENTION BEHAVIOUR OF SECOIRIDOID GLYCOSIDES ON SELECTED REVERSED-PHASE SYSTEMS

Column, 10-μm C_{18} Sil-X-10 with acetic acid modifier[a].

Compound	*Organic modifier*	*Log k_w*	*S*	*φ(%)*		*No. of points*	*Correlation coefficient*
				$k' = 1$	*$k' = 10$*		
Amarogentin	CH_3OH	3.2	5.6	56	39	6	1.000
1	THF	2.2	4.8	47	26	3	0.979
	CH_3CN	2.2	6.1	33	21	4	0.987
Amaroswerin	CH_3OH	3.1	5.7	56	36	6	0.995
2	THF	2.0	4.5	45	23	3	0.979
	CH_3CN	2.0	5.9	32	19	4	0.985
Gentiopicroside	CH_3OH	1.9	4.9	38	17	5	0.979
3	THF	0.8	4.4	17	>5	4	0.961
	CH_3CN	1.9	8.9	21	10	5	0.991
Common mean	CH_3OH		5.4 ± 0.44				
	THF		4.6 ± 0.21				
	CH_3CN		7.0 ± 1.7				

[a] Data are obtained according to eqn. 2.

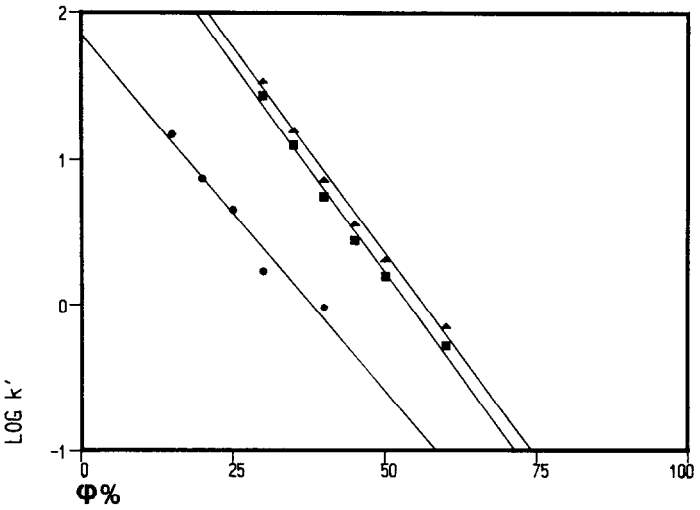

Fig. 2. Retention *vs.* methanol concentration (%, v/v; φ%) in the mobile phase. Column, 10-μm C_{18}, Sil-X-10 with acetic acid modifier. ▲ = Amarogentin; ■ = amaroswerin; ● = gentiopicroside.

CH_3OH < THF < CH_3CN, except for compound 3, where THF > CH_3CN (see Table I and Fig. 3). Thus, according to the LSS theory (eqn. 1) and provided that selected standard compounds are representative of such a natural extract, slow gradient programmes with restricted ranges are expected for CH_3CN, whereas faster gradients, operating within an extended $\Delta\varphi$ range, are more likely for CH_3OH. Intermediate conditions for both the $\Delta\varphi$ range and steepness rate are conceivable for THF.

Optimization of gradient elution conditions

The gradient was optimized with respect to the number of maxima in the chromatogram, allowing S/b to vary over a wide range, about four times the (S/b) values suggested by the LSS theory. S was taken as the mean solvent strength (see Table I) and b = 0.2 (10-μm C_{18} column). The $(S/b)_{LSS}$ values are 27, 23 and 35 for CH_3OH, THF and CH_3CN, respectively. According to previous experimental optimization trials performed on flavonoid plant extracts[1,5], the above-mentioned extended S/b range should contain the actual maximum.

TABLE II

ΔLOG k' VALUES[a] USING A 10-μm C_{18} SIL-X-10 COLUMN

Group contribution	*Compounds*	*Solvent*		*Δ log k'*
–OH	1–2	CH_3OH		−0.12 ± 0.01
		CH_3CN		−0.13 ± 0.01
		THF		−0.11 ± 0.02
$-C_{13}H_9O_4$	1–3	CH_3OH	(φ = 30%)	−1.32
		CH_3CN	(φ = 25%)	−1.32

[a] Differences between log k' values of the compounds listed.

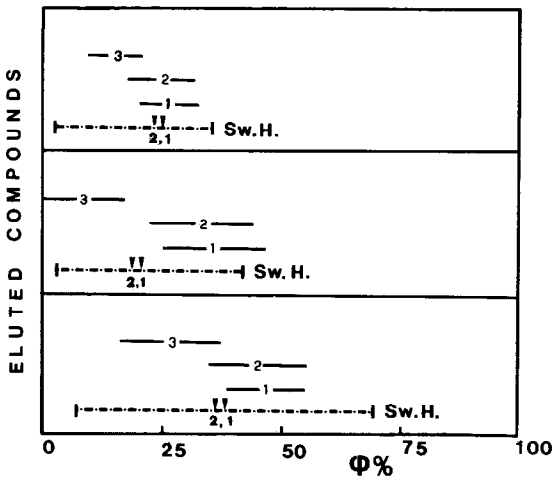

Fig. 3. Useful isocratic elution ranges, $\Delta\varphi$, where compounds exhibit $10 < k' < 1$ (solid lines), and gradient elution ranges for *Swertia* herb extract (Sw. H., dashed lines) with different organic modifiers (top, CH_3CN; centre, THF; bottom, CH_3OH). Gradient parameters correspond to optimum conditions. Columns: 10 μm C_{18} Sil-X-10 (isocratic data) and 10-μm C_{18} μBondapak (gradient data); flow-rate, 2 ml/min. For identified compound numbers (arrows), see Fig. 1.

In Table III, results of the procedures performed together with the theoretical LSS values are reported. Fig. 4 shows the optimization response curves. It can be seen that both the simplex (CH_3OH) and Fibonacci (CH_3CN and THF) approaches were able to attain a maximum. Hence the simplest Fibonacci approach is equally valid. However, it must be noted that the success of the Fibonacci approach was made

TABLE III

SEARCH FOR THE OPTIMUM GRADIENT

Column, 10-μm C_{18} μBondapak, flow-rate, 2 ml/min.

Run No.	*Organic modifier*					
	CH_3OH (simplex method)		*CH_3CN (Fibonacci method)*		*THF (Fibonacci method)*	
	S/b	*No. of peaks*	*S/b*	*No. of peaks*	*S/b*	*No. of peaks*
1	25	45	75	31	61	35
2	45	45	125	27	94	43
3	5	21	50	27	112	44
4	35	46	100	29	128	35
5	80[a]	35[a]	65	34	117	42
6	20[a]	31[a]	160[a]	25[a]	140[a]	32[a]
7	15[a]	25[a]	30[a]	26[a]	20[a]	26[a]
8	60[a]	40[a]				
LSS[b]	27 ± 3		35 ± 8		23 ± 1	

[a] Experimental points done in order to complete the response curve.
[b] *S/b* values predicted from the LSS theory.

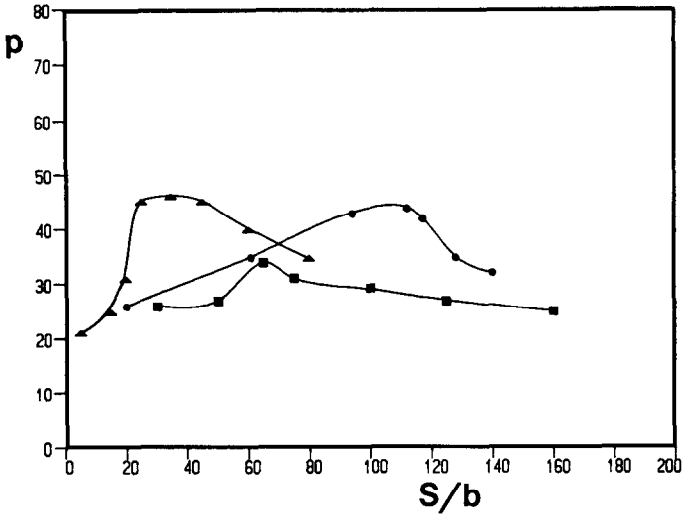

Fig. 4. Plots of number of peaks *vs.* S/b in gradient elution optimization with different organic modifiers (▲ = CH_3OH; ■ = CH_3CN; ● = THF). Data reported in Table III.

possible by appropriately setting up the search field, as it only works when the optimum search field contains an optimum point[17].

In Table IV, different features of optimum gradients obtained with the three solvents are reported. It can be seen that CH_3OH is the best organic modifier. In fact, it produces both the largest number of peaks (p = 46) and the highest peak-producing rate ($N_{c,k'}$ = 7.6) together with the shortest analysis time (τ_{tot} = 21) (see Table IV). The optimum gradient steepness, $(S/b)_{opt}$, is that closest to the $(S/b)_{LSS}$ value (their ratio is 1.3; see Table IV) and the total mixture is spanned over a very extended volume fraction value (0.63). CH_3CN produces the lowest total peak number but still shows a good peak-producing rate ($N_{c,k'}$ = 6.9) and the most favourable standardized analysis time (τ_{tot} = 19). Moreover, optimum gradient steepness is still related to the LSS theory. The $(S/b)_{opt}$ value is, in fact, $1.9(S/b)_{LSS}$ (see Table IV). THF produces a large number of peaks (p = 44) but with a very unfavourable analysis time (τ_{tot} = 43). The optimum gradient steepness is, in this last instance, far from the LSS value [$(S/b)_{opt}$ = 4.9 $(S/b)_{LSS}$, see Table IV] and unfavourable consequences are observed on separating skill ($N_{c,k'}$ = 3.0).

It must be noted that the $(S/b)_{opt}$ values differ for the three solvents. Moreover, they do not bear a simple relationship with the $(S/b)_{LSS}$ values, as would be expected according to the LSS theory. In this regard, the present results differ substantially from those observed previously with a natural flavonoid extract[1,5]. Any detailed explanation of the above features cannot be straightforward as the optimum obtained results from a complex interaction of not less than five basic factors: (1) gradient steepness, (2) gradient elution efficiency, (3) the type of response function undergoing optimization, (4) solvent strength towards mixtures of unknown components and (5) the retention time distribution of the different mixture components and its dependence on the mobile phase composition. The last factor may determine band reversal

TABLE IV

OPTIMUM GRADIENT FEATURES WITH DIFFERENT ORGANIC MODIFIERS

Column, 10-μm C_{18} μBondapak.

Organic Modifier	*No. of peaks, p (R_s = 0.5)*	*Gradient streepness, $(S/b)_{opt}$*	*Steepness ratio, $\frac{(S/b)_{opt}}{(S/b)_{LSS}}$*	*Organic modifier range, $\Delta\varphi$*	*Standardized time' τ_{tot}*	*Peak capacity, N_c*	*Peak-producing rate, $N_{c,k'}$*
CH_3OH	46	35	1.3	0.63	21	160	7.6
CH_3CN	34	65	1.9	0.31	19	131	6.9
THF	44	112	4.9	0.39	43	128	3.0

effects and multiple maxima[21]. Unfortunately, the last two factors are largely unpredictable.

It can be observed that all the three response functions exhibit more or less distinct maximum regions (see Fig. 4). This can be explained if one remembers that whenever the gradient steepness is lowered, the resolution is enhanced because of the increase in the mean capacity factor (as fully explained in ref. 3). However, at the same time, the peak heights are lowered. This last factor has an opposite effect to the observed number of peaks. In fact, not only must they be produced by setting up a stronger resolving power, but they must also be detected above a fixed critical noise level. With respect to this mechanism, which probably produces the well behaved maximum in the optimization plot as for CH_3OH and THF (see Fig. 4), CH_3CN apparently differs: the maximum region is flattened with only a weakly defined maximum (see Fig. 4). The only explanation that can be offered for this behaviour is the different overall selectivity of CH_3CN in comparison with those of CH_3OH and THF. In fact, CH_3CN has the highest eluting power (see Table I) and the $\Delta\varphi$ ranges spanned for gradient elution are accordingly the narrowest (see Table IV and Fig. 3). Therefore, even if $N_{c,k'}$ (an intensive quantity) is still high, both the total peak capacity N_c and the total number of peaks produced p (extensive quantities) are small. Under these conditions, the narrow chromatographic space was always too crowded. Hence those mechanisms giving variations in peak number were unable to produce a well extended and pronounced maximum response.

A few comments must be made regarding the relationship between the experimental and LSS optima. It has been observed that whenever these quantities are far apart, the peak-producing rate suffers, as with THF (see Table IV). Therefore, it appears that if the experimental optimum had not been found close to the value given by the LSS theory, it would not have been a good one. Nonetheless, how can we explain this anomalous THF behaviour?

The results with THF were analysed in detail in order to find correlations among some of the features. A pronounced, steady increase in peak number was observed as long as the S/b values were increasing, mainly in the initial part of the chromatogram. On the other hand, if we refer to the less retained test compound (gentiopicroside), THF had such a strong eluting power that, even at $\varphi = 0.05$, k' was only 3.4. It must therefore be concluded that a large number of components of a mixture behaving in this way can best be resolved by just setting up an initial isocratic

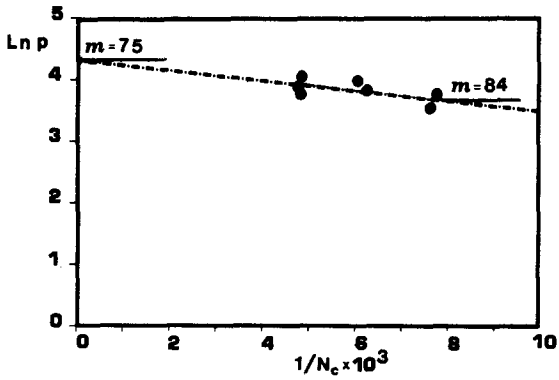

Fig. 5. Davis–Giddings plot for extract of *Swertia* herb. Experimental conditions are reported in Table V.

elution. Once in the gradient optimization mode, they will direct the search towards slow programmes derived by high S/b values. This is what was effectively observed. For the same reason, only with THF are the φ values at which amarogentin and amaroswerin elute in the gradient mode far from the values at which the same compounds have $k' = 10$ in the isocratic mode (see Fig. 3). The explanation is that, with these slow THF gradients, the test solutes start and continue to migrate at low φ values until they reach the end of the column. These gradient elution conditions are similar to those of true and proper isocratic runs with high k' values. Hence they are different from the best conditions described by the LSS theory[3,4]. The lowest $N_{c,k'}$ value observed in an optimum THF gradient (see Table IV) is therefore coherent with the above picture. In conclusion, it must be asserted that the overall THF optimization was determined by local effects, although the LSS theory is able to explain these results.

Evaluation of number of components by the Davis–Giddings theory

In order to establish the effective extent of separation (eqn. 4) obtained, the number of component m was evaluated by the Davis–Giddings procedure[1,6] (see Fig. 5 and Table V). Eqn. 3 was employed to determine the number of components in a

TABLE V

EXPERIMENTAL CONDITIONS OF THE DAVIS–GIDDINGS PLOT DATA

Organic modifier	*Column*[a]	*Flow-rate (ml/min)*	*p*	*Ln p*	$1/N_c \cdot 10^3$	N_c
CH_3OH	1	1	48	3.871	4.76	210
CH_3OH	2	1	56	4.025	4.85	206
CH_3OH	2	2	53	3.970	6.06	165
CH_3OH	1	2	46	3.828	6.25	160
CH_3OH	2	2	42	3.761	4.85	206
THF	1	2	43	3.738	7.78	128
CH_3CN	1	2	34	3.526	7.62	131

[a] 1, 10-μm C_{18} μBondapak; 2, 5-μm C_{18} Bakerbond.

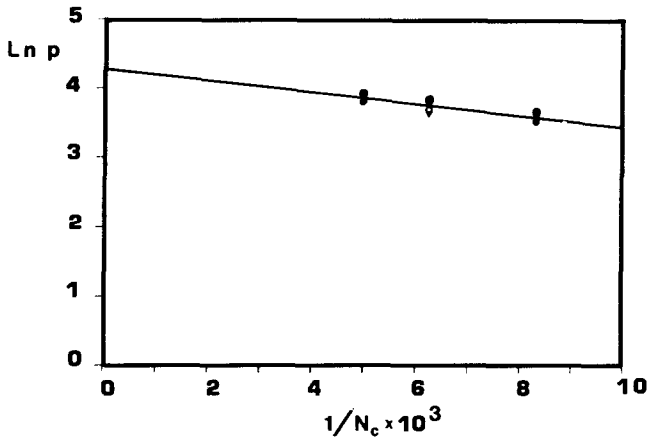

Fig. 6. Davis–Giddings plot of simulated chromatograms at various peak capacities. Identical symbols correspond to the same random sequence of retention times. Full line, calculated line of $\ln p = \ln m - m/N_c$ for $m = 70$.

Swertia herb extract. Two different columns, with 5- and 10-μm particles, and several organic modifiers were used to allow the peak capacity N_c to vary by different flow-rates (see Table V). All these data refer to chromatograms obtained under optimum separation conditions. All of them have a Poisson retention time distribution. Although there are few points in the Davis–Giddings plot and they are scattered, they single out a common m value (*ca.* 80) from both the intercept ($m = 75$) and the slope ($m = 84$).

These results were validated by using the same simulation procedure discussed in ref. 1. Here, three different Poisson-like retention sequences having random heights were generated as three organic modifiers giving different retention sequences were used. Chromatograms with different peak capacities were simulated with each sequence. The Giddings–Davis plot shown in Fig. 6 was obtained by using those simulated chromatograms. It can be seen that there are two sources of variability around the theoretical straight line, one ascribed to the effect of changing peak capacities and the other due to the different random retention time sequences. Overall, the simulation plot qualitatively resembles that derived from experimental data (Fig. 5), hence the latter is properly validated. The experiments reported in Fig. 5 are, to our knowledge, the first example of a Davis–Giddings plot obtained in LC by using both different columns and organic modifiers.

Evaluation of optimum performance

An example of an optimum chromatographic separation obtained on a 5-μm C_{18} column using CH_3OH as organic modifier is shown in Fig. 7. The values of the chromatographic parameters for $R_s = 0.5$ are $N_c = 165$, $p = 53$, $m = 80$, $\gamma = p/m = 0.7$ and $\alpha = m/N_c = 0.48$. If a more stringent resolution criterion is assumed, *e.g.*, $R_s = 1$, the peak capacity is lowered (by about 50%) and so also is the peak number, which may be evaluated as $p = 30$ by using eqn. 3. Among these peaks at $R_s = 1$, the Davis–Giddings theory predicts twelve singlets, *i.e.*, those peaks with a purity of

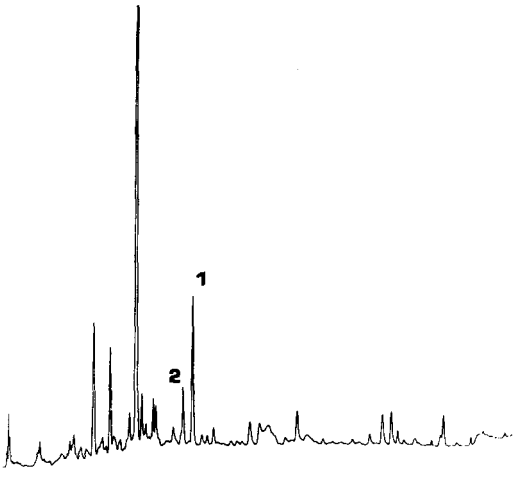

Fig. 7. Example of optimum separation on a 5-μm C_{18} Bakerbond column with methanol and acetic acid modifiers. Gradient elution programme refers to run No. 3 in Table V. 1 = Amarogentin; 2 = amaroswerin.

about 95%. Moreover, the likelihood that, among 30 peaks, one or two randomly chosen will all be singlets is 40% and 16%, respectively. A peak purity check was performed by spectroscopic analysis on identified amarongetin and amaroswerin peaks (chromatogram in Fig. 7). It was found that only one of these two peaks was a single component, consistent with the above.

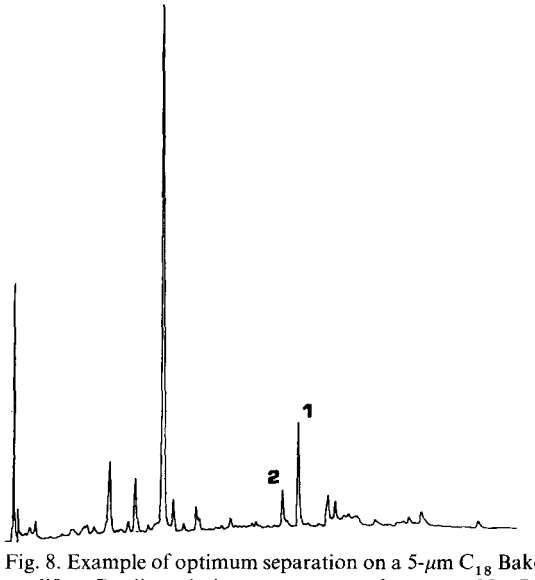

Fig. 8. Example of optimum separation on a 5-μm C_{18} Bakerbond column with acetonitrile and acetic acid modifier. Gradient elution programme refers to run No. 7 in Table V. 1 = Amarogentin; 2 = amaroswerin.

The quality of the optimum separation (Fig. 7) was evaluated by calculating β (eqns. 9 and 10). Assuming $R_s = 1$, then $\beta = 0.14$, which is 22% lower than the maximum allowable value (0.18)[6]. Hence, for such a complex mixture, the best performed optimization (CH_3OH case) approaches the absolute optimum and might be considered fairly good from such a point of view. On the other hand, more singlet peaks could be obtained not only by means of more efficient columns, but also by the combined use of two or more gradient separations. This can be understood by comparing the separation obtained with CH_3OH (Fig. 7) with one of those obtained with CH_3CN (Fig. 8). The overlapping patterns appear slightly different, once substructural peak cluster distributions have been compared. Thus, although the number of singlets is expected to decrease on going from CH_3OH to CH_3CN, the chemical identities of the singlets produced might be different. In this instance the combined use of such gradients would increase the total number of individual singlets separated and, consequently, the amount of information gained. Nevertheless, a quantitative evaluation of this general improvement would be beyond the scope of this paper.

CONCLUSIONS

The previously reported gradient optimization runs, as for unknown multicomponent mixtures, always gave gradient steepness values that were about twice those suggested by the LSS theory[1,6]. Nonetheless, the present results indicate that there are actually many largely unpredictable outcomes possible. However, the combined use of the LSS theory and the statistical peak overlap theory of Davis and Giddings are able to evaluate the quality of an attained optimum. Hence automatic optimization methods, which are powerful and irreplaceable practical techniques, have to be critically employed according to the above-mentioned chromatographic theories. These methods should also be developed in order to incorporate properly more advanced, sophisticated utility functions with a strong theoretical background.

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SYMBOLS

b gradient steepness constant;
m number of components in the mixture above a defined concentration (about 0.3% of the most abundant component); it is defined by the Davis–Giddings method;
k' capacity factor;
k_w extrapolated value of k' for $\varphi = 0$ (eqn. 2);
p number of peaks appearing in the chromatogram at a given resolution R_s;
s number of singlet peaks (eqn. 7); it is the number of peaks containing a single component; it is not an experimental quantity but is evaluated by using the Davis – Giddings theory[6];
t_o retention time of an unretained compound;
t_{tot} total gradient analysis time (eqn. 4);

x_f last peak position in the chromatogram;
x_i first peak position in the chromatogram;
x_o mean peak width at the baseline;
N_c peak capacity at a given resolution (eqns. 5 and 6);
$N_{c,k'}$ peak capacity produced per unit capacity factor k'; it is the maximum number of single peaks which can be contained in a unit k' value under a given gradient elution run; the resolution assumed is $R_s = 0.5$;
R_s resolution between adjacent peaks;
α m/N_c (eqn. 8), saturation factor; it is a measure of saturation of the chromatographic space;
β s/N_c (eqn. 9), singlet peak saturation factor;
γ extent of separation, p/m; it is the degree of separation of the mixture obtained (eqn. 7);
τ_{tot} standardized total analysis time of the gradient elution expressed in k' units (eqn. 3); it is an adimensional quantity;
φ volume fraction of organic modifier in the mobile phase;
φ_f final volume fraction of organic modifier corresponding to the last peak position in the chromatogram;
φ_i initial volume fraction of organic modifier corresponding to the first peak position in the chromatogram.

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