Journal of Chromatography A, 658 (1994) 195–205
Elsevier Science B.V., Amsterdam

CHROM. 25 158

Review

Gradient elution in preparative reversed-phase liquid chromatography

G. Cretier* and J.L. Rocca

Laboratoire des Sciences Analytiques, Université Claude Bernard–Lyon I, 43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cedex (France)

ABSTRACT

Reversed-phase gradient elution is becoming a method of choice for the laboratory-scale preparative separation of complex mixtures and for the industrial-scale purification of biopolymers. Understanding of band broadening under overloaded conditions in gradient elution is also developing and many papers based on the simulation of elution profiles have recently been published. They are reviewed with emphasis on the resulting optimization schemes for gradient elution preparative separations. The specific problems connected with the preparative chromatography of peptides and proteins under gradient elution conditions are not discussed.

CONTENTS

1. INTRODUCTION

Gradient elution is a commonly used technique for analytical separations in reversed-phase liquid chromatography. Its major areas of application are the analyses of complex mixtures and the separations of biopolymers (peptides and proteins). For complex mixtures of components that exhibit a broad range of retentivity, the use of gradient elution allows the separation of the whole sample in a single run: weakly retained components are eluted with the mobile phase of lower elution strength in the first step of the

* Corresponding author.

0021-9673/94/$26.00
SSDI 0021-9673(93)E0508-R

gradient run whereas strongly retained components are eluted with the mobile phase of relatively higher elution strength in the last step of the gradient run. For the separation of large biomolecules in reversed-phase liquid chromatography, gradient elution is almost inevitable. Desorption of the components from the apolar stationary phase requires a given amount of organic modifier in the aqueous mobile phase [1]: before this critical mobile phase composition, the adsorption of the biopolymer on the stationary phase is almost irreversible; beyond this point, the interaction with the stationary phase becomes very small and the component moves rapidly through the column while being repeatedly partitioned on the stationary phase. Hence elution of all biopolymers with a single mobile phase composition (isocratic elution) is nearly impossible whereas a progressive increase in the eluent strength during the gradient elution allows the successive desorption of the components that are initially strongly bound to the stationary phase.

Gradient elution is becoming a preparative technique which is being widely investigated. It is especially attractive for laboratory-scale separations where the usual objective is to obtain a certain amount of product with a specified purity, within a certain recovery ratio limitation, with the expenditure of both minimum time (*i.e.*, achieving maximum production rate) and development effort, with given equipment and without cost consideration. On the industrial scale, application of gradient elution is an expensive procedure: first, washing and regenerating the column after the elution of the peak of interest and prior to the next operation are time consuming and require large volumes of solvents; second, solvent regeneration for recycling is more complex and expensive with a constantly changing mobile phase composition than with a constant composition in the isocratic elution mode. Hence the application of large-scale gradient elution reversed-phase liquid chromatography seems to be limited to the purification of biopolymers which cannot be carried out in another way. For large-scale recoveries of small molecules from complex mixtures containing weakly and strongly retained solutes, there are some other less expensive possibilities instead of gradient elution, such as trapping strongly retained impurities on a precolumn or performing flip-flop elution, which can be considered as an optimized backflush operation [2].

The quality of the preparative separation of a binary mixture by gradient elution is affected by a large number of experimental variables and, in order to have a better understanding of how the separation varies according to the experimental parameters, the usually adopted approach was the computer simulation of elution band profiles [3–10]. In fact, owing to the non-linearity resulting from the multi-component adsorption behaviour under overload conditions, no analytical solution of the process equations exists and calculation of band elution profiles requires efficient numerical techniques. Two basically different algorithms were used: the Craig machine [3,4,6–9] and the discretization of the mass balance equation using the orthogonal collocation method on finite elements [5,10]. These two algorithms allow the finite efficiency of the column to be simulated. The most widely simulated gradient elution mode was linear gradient (linear change of mobile phase composition with time) [3–9]; only one study was dedicated to the theoretical investigation of step elution mode [10]. In order to simplify the procedures, all these models assumed that multi-component adsorption data can be fitted by the competitive Langmuir isotherm equation [11]. For large biomolecules such as proteins whose conformation and, consequently, adsorption can be significantly altered by any change in the organic modifier content of the mobile phase, this isotherm model seems to be very unrealistic [12]. For small molecules, the agreement between the experimental and the calculated elution band profiles for a single component was shown to be satisfactory only for both moderate column loading and reasonable gradient steepness [13]; for higher overload or for steeper gradient, the agreement deteriorates because, in these instances, owing to the concentrating effect during the gradient elution, the solute concentration of the migrating band may reach very high values and the Langmuir isotherm equation is no longer suitable to fit accurately the isotherm data within

a wide concentration range, especially in the high concentration zone of the curve [14].

Consequently, the published models allowing the simulation of a preparative gradient elution separation are not yet quantitative. However, most of the features exhibited by these theoretical studies were qualitatively confirmed by experiments [4,6,15–18]. Therefore, this investigation by simulation provides an interesting insight into the phenomena governing gradient elution separation under overload conditions and the derived conclusions can be considered as useful guidelines for the selection of the best experimental conditions in preparative gradient elution reversed-phase liquid chromatography.

2. DESIGN PARAMETERS IN LINEAR GRADIENT ELUTION MODE

The analytical separations carried out in reversed-phase liquid chromatography under linear gradient conditions are accurately predicted by the linear solvent strength (LSS) theory [19]. This theory is based on the fact that, in isocratic elution reversed-phase liquid chromatography, the variation of the retention of a solute j can be reasonably described by an empirical linear relationship between the logarithm of the solute capacity factor k'_j and the volume fraction of modifier in the mobile phase, φ:

$$\log k'_j = \log k'_{w,j} - S_j\varphi \quad (1)$$

where $k'_{w,j}$ represents the capacity factor of solute j with pure water as mobile phase ($\varphi = 0$) and S_j is a constant depending on both the solute and organic modifier used in the organic–water mobile phase. In order to optimize the analytical separation of a mixture of two solutes 1 and 2, it is essential to determine the two log k'_j *versus* φ plots [20,21].

In isocratic elution, the S_1 and S_2 values influence the variation of the separation factor α (or selectivity, defined by $\alpha = k'_2/k'_1$) with the mobile phase composition φ:

$$\log \alpha = \log\left(\frac{k'_{w,2}}{k'_{w,1}}\right) - (S_2 - S_1)\varphi \quad (2)$$

According to whether $S_1 = S_2$, $S_1 > S_2$ or $S_1 < S_2$, the log k'_j *versus* φ plots are parallel (Fig. 1a), divergent (Fig. 1b) or convergent (Fig. 1c) and the separation factor α remains constant, decreases or increases with decrease in φ, respectively. As suggested by Antia and Horvath [5], in the following these three cases can be denoted parallel, divergent and convergent solutes.

The LSS theory [19] shows that, in gradient elution, separation can be also inferred from the isocratic plots of log k'_j *versus* φ. Band spacing, defined as the difference between the analytical retention times $t_{R,1}$ and $t_{R,2}$, is related to the mobile phase compositions $\varphi_{f,1}$ and $\varphi_{f,2}$ at the outlet of the column when solutes 1 and 2 are eluted, according to

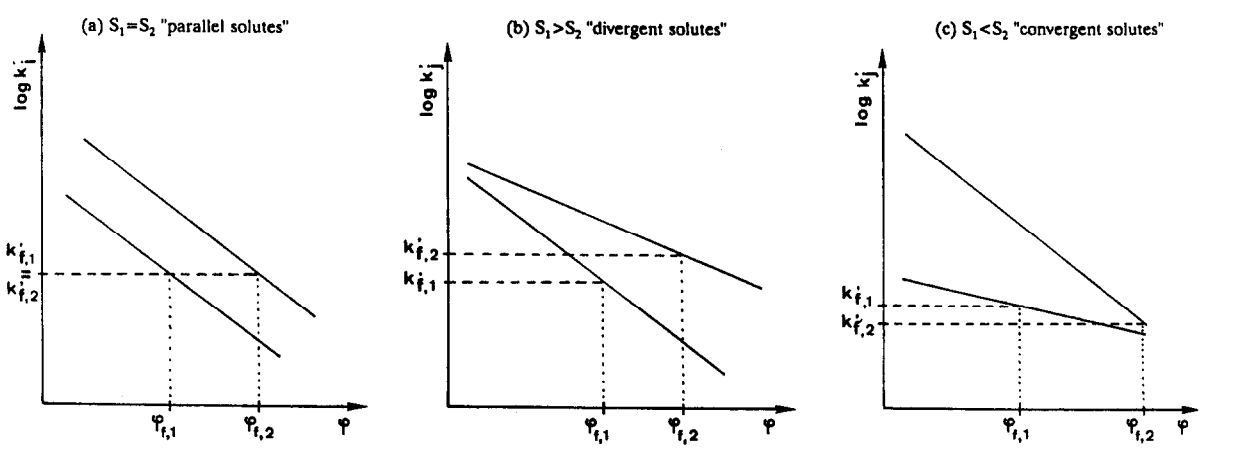

Fig. 1. Representation of gradient elution in reversed-phase liquid chromatography from relationships between isocratic capacity factor k'_j and mobile phase composition φ (see text).

$$t_{R,2} - t_{R,1} = \frac{\varphi_{f,2} - \varphi_{f,1}}{\Delta\varphi / t_G} \quad (3)$$

where $\Delta\varphi$ is the change in modifier volume fraction during the gradient and t_G is the gradient time; $\Delta\varphi/t_G$ is the gradient slope. These final mobile phase compositions $\varphi_{f,j}$ (vertical dotted line in Fig. 1) correspond to the isocratic capacity factor $k'_{f,j}$ (horizontal dashed line in Fig. 1) given by

$$k'_{f,j} = \frac{1}{2.3 S_j (\Delta\varphi / t_G) + \dfrac{1}{k'_{0,j}}} \quad (4)$$

where $k'_{0,j}$ is the capacity factor of solute j for the initial mobile phase composition φ_0 when the gradient run is started. If $S_1 = S_2$, the two bands leave the column with the same instantaneous capacity factor ($k'_{f,1} = k'_{f,2}$).

If the gradient slope or the elution strength of the initial mobile phase is decreased (smaller $\Delta\varphi/t_G$ or smaller φ_0), the final capacity factors $k'_{f,1}$ and $k'_{f,2}$ increase in all instances (eqn. 4), but the difference between the final mobile phase compositions, $\varphi_{f,2} - \varphi_{f,1}$, remains constant when $S_1 = S_2$ (parallel solutes, Fig. 1a), decreases when $S_1 > S_2$ (divergent solutes, Fig. 1b) and increases when $S_1 < S_2$ (convergent solutes, Fig. 1c). Consequently, it can be concluded from eqn. 3 that, on the one hand, the band spacing $t_{R,2} - t_{R,1}$ is an inverse function of the gradient slope $\Delta\varphi/t_G$, getting steeper and steeper from divergent solutes to parallel solutes and to convergent solutes, respectively; on the other hand, the band spacing does not depend on the initial mobile phase composition φ_0 for parallel solutes, decreases by decreasing φ_0 for divergent solutes and increases by decreasing φ_0 for convergent solutes.

3. BAND BROADENING IN OVERLOADED GRADIENT ELUTION

3.1. Single solute

Fig. 2 compares single peaks obtained for different loads in isocratic elution and in gradient elution with various gradient slopes. Here, the column loading and gradient slope are respectively characterized by the following classical dimensionless parameters: the loading factor L_j, defined as the ratio of the injected amount of solute to the amount of solute adsorbed on the stationary phase at column saturation (column saturation capacity), and the gradient steepness G, related to the gradient slope by [19]

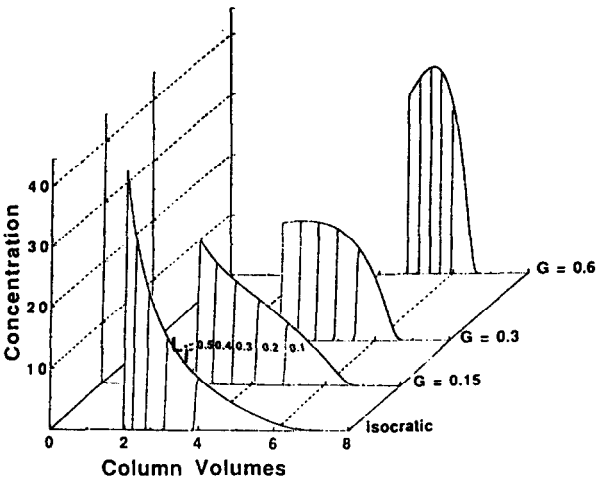

Fig. 2. Effect of elution conditions and column loading on the peak profile of a single component (from ref. 5). Peaks were simulated for different loading factors L_j and different gradient steepnesses G (see text). Gradient conditions (slope and initial mobile phase composition) were adjusted in order to obtain the same elution volume of the band tail as in isocratic elution.

$$G = (\Delta\varphi / t_G) t_M S_j \quad (5)$$

where t_M is the column dead time.

Whatever the elution mode, the fronts of the overloaded peaks become sharper and sharper and elute progressively earlier with increasing concentration injected. On the other hand, the peak tails remain coincident and elute at the analytical retention volume whatever the solute load. This band broadening results from non-linearity of the solute distribution isotherm at high concentration. The elution mode influences the shape of the band rear envelope. In isocratic elution, the peak shows an extended concave upward rear profile. In gradient elution, the peak is compressed: the mobile phase is always stronger at the peak rear than at the peak front

and, consequently, the velocity of the peak rear is always greater than that of the peak front; this compressive effect tends to compensate the band broadening resulting from isotherm non-linearity and the rear of the peak exhibits a rounded concave downward shape.

The variations of band broadening for a one-component sample as a function of sample size $Q_{i,j}$ and gradient conditions were widely studied from both simulated [3,4,6] and experimental [4,6] peaks. If, according to the Knox treatment [22], the baseline width W_j of the overloaded band is assumed to be the sum of contributions coming from column dispersion (measured by the baseline width of the analytical peak, $W_{0,j}$) and column overload $W_{th,j}$:

$$W_j^2 = W_{0,j}^2 + W_{th,j}^2 \quad (6)$$

the mass-related contribution to the band width $W_{th,j}$ was shown to be approximately described by

$$W_{th,j} = \frac{\text{constant}}{(\Delta\varphi/t_G)S_j}(Q_{i,j})^z \quad (7)$$

with $0.5 < z < 0.6$.

Although eqns. 6 and 7 are valid only for a single component, they can be used to guide the design of "touching band" separations of closely eluted compounds, separations for which small injected amounts of solutes lead to relatively weak interferences between the migrating species. This situation is illustrated schematically in Fig. 3:

$$W_2 = t_{R,2} - t_{R,1} \quad (8)$$

and, from eqns. 6 and 7, the injectable amount is written as

$$Q_{i,2} = \frac{(\Delta\varphi/t_G)^{1/z} S_2^{1/z}}{(\text{constant})^{1/z}} [(t_{R,2} - t_{R,1})^2 - W_{0,2}^2]^{1/2z} \quad (9)$$

This approach is used as the basis of the commercially available computer program BIOPREP for the development of reversed-phase gradient-elution high-performance liquid chromatographic separations of peptide and protein samples [15,23]. Using eqn. 9 with $z = 0.55$, this program calculates the sample size and the corresponding

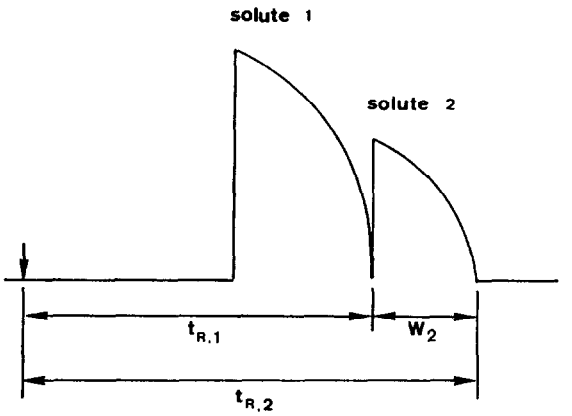

Fig. 3. Schema of a "touching band" separation.

production rate for touching band separation as a function of gradient time. This considerably simplifies the development of preparative separations for maximum throughput of purified material. Only three preliminary experimental runs are required for the calculations. First, two analytical runs are carried out with different gradient times in order to determine the solute retention characteristics S_j and $k'_{w,j}$ (see refs. 20 and 21) and to make possible the further estimation of the variations of retention times $t_{R,1}$ and $t_{R,2}$ and band width $W_{0,2}$ as a function of gradient time (see ref. 19). Second, one of the gradient runs is repeated by injecting a higher sample amount in order to determine the values of the constant in eqn. 9. These experiments and the further study of the injectable amount and production rate *versus* gradient time variations can be carried out with a small-sized column. In order to increase the production rate, BIOPREP scales up the optimum gradient conditions and sample load for a column of larger size packed with the same stationary phase, keeping constant the gradient steepness G and increasing the sample size in proportion to the column volume increase.

3.2. Binary mixtures

3.2.1. Governing phenomena

In gradient elution, as in isocratic elution, the peak elution profiles of a two-component mixture are controlled by two phenomena: the

displacement and the tag-along effects [5,7–10,16–18]. These effects have been investigated in the isocratic elution mode [18,24–32]. They are the consequences of the competitive access by the molecules of the closely eluting components to the stationary phase. Hence they are very strong in "band overlapping" separations for which the recovered amount and the production rate are increased at the expense of product recovery. The first effect, the displacement effect, is beneficial for the recovery of large amounts of pure first solute: the molecules of the second solute interact more strongly with the stationary phase and displace the molecules of the first solute from the stationary phase, so the more retained solute tends to push ahead the less retained solute; then, the main part of the first solute is eluted as a narrow, concentrated and pure band in front of the second solute. The displacement strength of solute 2 increases with its increasing, on the one hand, relative concentration in the sample, and on the other, relative affinity for the stationary phase *i.e.*, the separation factor α. The second effect, the tag-along effect, influences adversely the separation and takes place only with a weak displacement effect. When the concentration of the first component is high, solute 1 molecules tend to crowd the solute 2 molecules out of the stationary phase; this effect is more and more pronounced as the affinities of the two solutes for the stationary phase become closer and closer, *i.e.*, as the separation factor α becomes lower and lower. The front of the second component band then moves faster than it would do if it was pure, leading to a wider corresponding elution peak.

3.2.2. *Effect of the sample*

Figs. 4 and 5 show that, in the gradient elution mode, the relative intensities of the displacement and tag-along effects also depend on the composition of the feed and, further, on the S_j values of the two solutes. For two sample compositions (1:9 and 9:1), Fig. 4 superposes the band profiles corresponding to injection of the mixture (thick line) and to injection of the equivalent amounts of single solutes (fine line). In the case of the 1:9 mixture (Fig. 4a), the more retained solute constituting the main part of the sample strongly

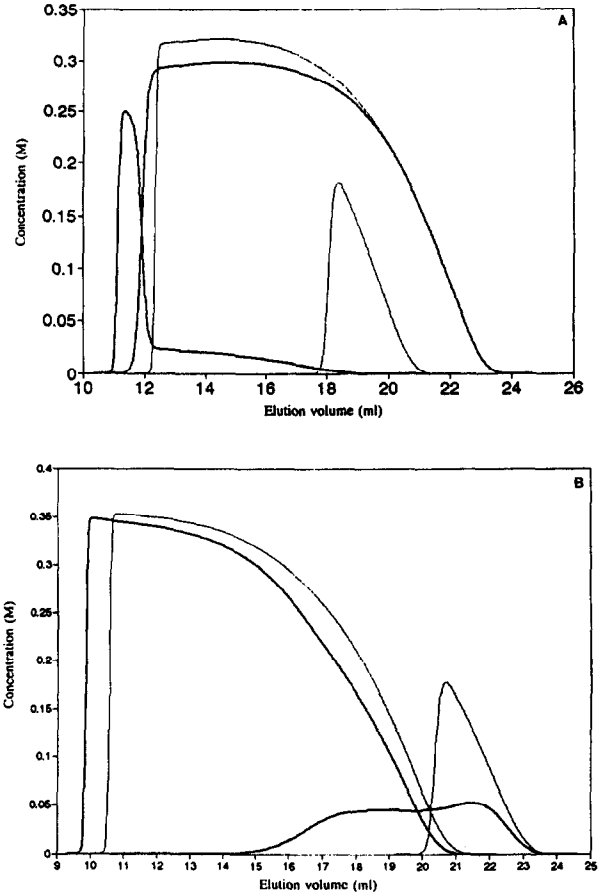

Fig. 4. Chromatograms simulated by CRAIGSIM and corresponding to two different relative compositions of a mixture of parallel solutes (from ref. 33). (a) 1:9 mixture; (b) 9:1 mixture. Sample load: 2.96 mmol. Sample concentration: 1.7 mol l^{-1}. Thick line, band profiles obtained by direct injection of the mixture; fine line, band profiles corresponding to equivalent amounts of single solutes.

displaces the less retained solute that is eluted with a characteristic L-shaped band. The excrescence that appears at the front of the first component is eluted at a concentration level (0.25 mol/l) higher than the solute feed concentration (0.17 mol/l). With the 9:1 mixture (Fig. 4b), the displacement effect of the major first component is hardly noticeable and the tag-along effect of the minor second component is very pronounced; its migration is significantly accelerated when the two solutes co-elute and the bands considerably overlap.

Fig. 5 compares the effects of increased sam-

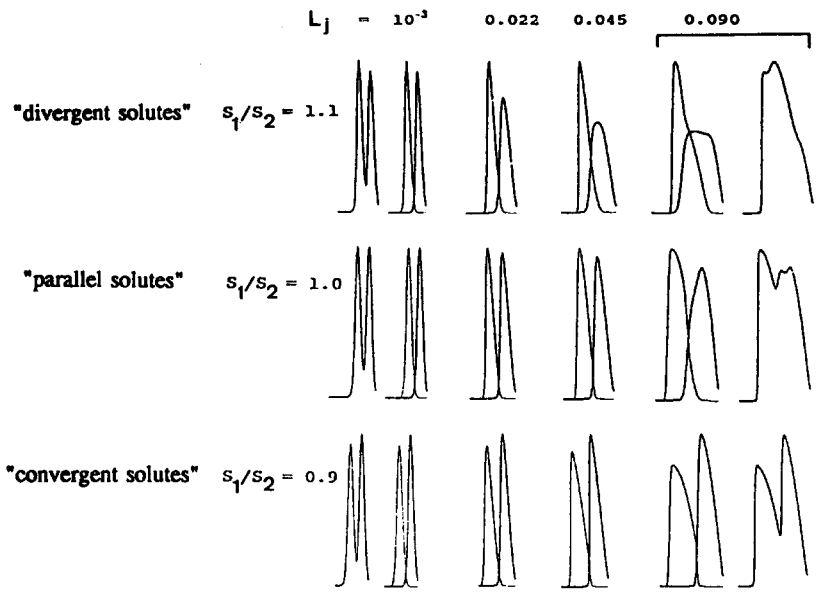

Fig. 5. CRAIG4 simulations for the separation of different equimolar mixtures (different values of S_1/S_2) as a function of sample size (loading factor L_j) (from ref. 8). Gradient conditions were selected to give equal analytical resolution for a 10^{-3} loading factor (see text).

ple size (loading factor L_j) for separation of different equimolar mixtures of divergent, parallel and convergent solutes. In all instances, the gradient steepness G was kept constant and the composition of the initial mobile phase was adjusted in order to give the same analytical resolution when a small amount of sample ($L_j = 10^{-3}$) is injected. As sample size is increased, the separation for the mixture of divergent solutes ($S_1/S_2 = 1.1$) deteriorates very rapidly and, for $L_j = 0.09$ (last chromatogram), the recovery of 99% pure solute 2 is only 28%. This reflects the fact that, for divergent solutes, the initial values of the instantaneous separation factor α are small. Consequently, during the first step of the gradient run, the separation of solutes is moderated and partly counterbalanced by a strong tag-along effect that is taking place due to the small α values. For the mixture of convergent solutes ($S_1/S_2 = 0.9$), the bands are totally resolved regardless of sample size; the recovery of the 99% pure solute 2 is about 99% for any of the investigated sample amounts. The reason is that, for convergent solutes, the larger initial values of the instantaneous separation factor α and the concomitant strong displacement effect lead to an efficient separation of solutes as the gradient run is started. These examples demonstrate that, in gradient elution, the loading capacity of the column is greatly dependent on the S_j values for the sample to be separated.

3.2.3. *Effect of injection conditions*

In most instances, the sample is dissolved in the mobile phase composition corresponding to the initial composition of the gradient; consequently, for large sample volumes, the front of the injected band starts to move under isocratic conditions and the band profiles can be significantly delayed compared with the elution profiles obtained for a Dirac plug injection. Further, for a given injected amount of sample, the choice of injection volume sets the injected solute concentrations and, in conjunction with the concentrating effect of the gradient, the maximum concentrations of the solutes in the column. Knowing that, for high sample loads, the chromatographic peak profiles are greatly influenced by the adsorption behaviour of the highly concentrated solute, the injection conditions play an important role in determining the band profiles [9,10,14,18,33]. The attempts to

model overloaded gradient elution separations for multi-component samples often ignored this issue and, regardless of the role of injection conditions, assumed that only the magnitude of the sample load has to be taken into account [3–8].

The general rule is that a small volume of a concentrated feed solution has to be used rather than a large volume of a diluted feed solution [10,33], except when the tag-along effect is predominant [9,18,33]. A similar conclusion was reported in isocratic elution preparative chromatography [33,34]. When the tag-along effect is very strong, for example, when the substance to be recovered is both the main part of the sample and the first-eluted solute, the amount of pure substance recovered from the injection of a given sample amount is maximum for an optimum value of the injected concentration [9,18,33]. This optimum concentration results from a compromise between two competitive effects (Fig. 6): when the injected concentration is increased

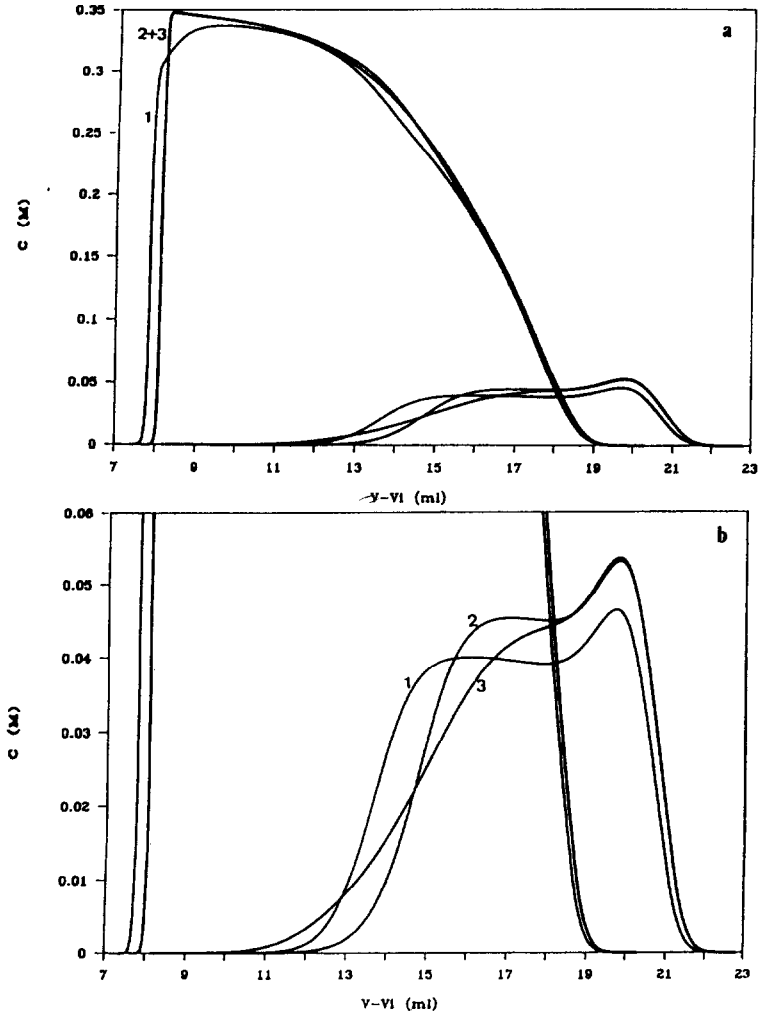

Fig. 6. Three chromatograms simulated by CRAIGSIM and corresponding to the same sample load and three different injection concentrations (from ref. 9). Mixture of parallel solutes: relative composition = 9:1; injected amount = 2.96 mmol. (a) Global chromatograms; (b) magnified second-eluted peaks (the elution volume V is adjusted by subtracting the injection volume V_i). Elution profiles: (1) injection concentration $C_i = 0.1$ mol l^{-1}, recovery of the 99% pure first solute $r_1 = 72.6\%$; (2) $C_i = 1.7$ mol l^{-1}, $r_1 = 81.0\%$; (3) $C_i = 27.8$ mol l^{-1}, $r_1 = 72.3\%$.

and the injected volume is decreased (the injected amount being constant), classically the peak width is decreased, the separation is improved and the first component recovery is increased (comparison of elution profiles 1 and 2); however, simultaneously, the tag-along effect becomes stronger and the front base of the second component is gradually attracted under the first component peak; at a high injection concentration, this additional band broadening finally becomes predominant and is responsible for the decrease in the first component recovery (comparison of elution profiles 2 and 3).

3.2.4. *Effect of column plate number*

In gradient elution [7,10,17], the influence of column efficiency is similar to that observed in isocratic elution [26]: as shown by the variation of the recoveries of 99% pure solutes from a constant sample size with increasing column plate number N (Fig. 7), separation initially increases with increasing N and then levels off. A high column efficiency sharpens the peak edges, maximizes the displacement effect and minimizes the tag-along effect: the displaced peak front of the first solute has a much narrower width and a much higher concentration; the peak front of the second solute becomes more vertical; its bottom and its top tend to co-elute with the same concentration of the first solute and they are subjected to consecutive tag-along effects of close intensities, contributing to reducing the front base broadening that is detrimental to the recovery of pure first solute.

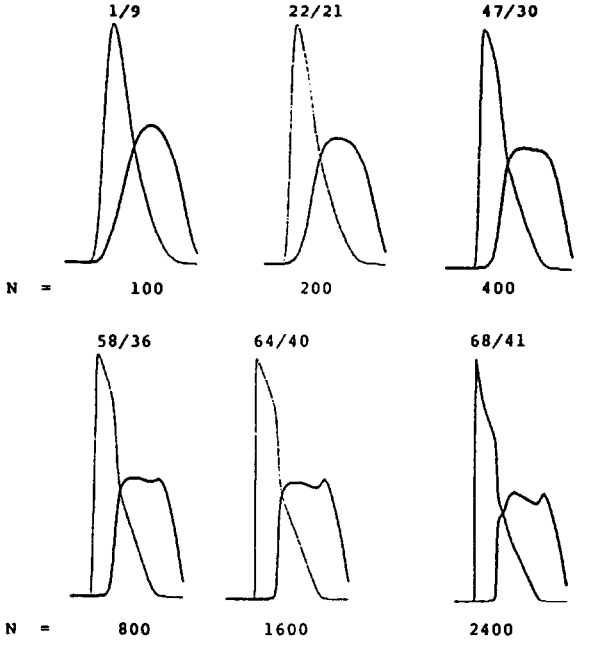

Fig. 7. CRAIG4 simulations for the separation of an equimolar mixture of two parallel solutes as a function of column plate number (from ref. 7). Loading factor $L_j = 0.40$. Recoveries of 99% pure solutes are indicated above each chromatogram.

Because of the significant reduction of the tag-along effect with increasing theoretical plate number, the optimum injection concentration for major/minor mixtures is an increasing function of column efficiency [9].

3.2.5. *Effect of gradient conditions*

The gradient slope has a relatively small effect on the displacement and tag-along effects. Consequently, preparative separation of a given sample size simply follows the evolution of analytical separation and generally increases by decreasing the gradient slope, this decrease being faster for mixtures of convergent solutes than for mixtures of divergent solutes [7,8,16,17]. On the other hand, the choice of the initial mobile phase composition φ_0 significantly affects band broadening during the sample injection and, thus, the final separation [7,8,16]. For mixtures of convergent solutes, a decrease in φ_0 is very beneficial owing to an improvement in the analytical separation and an enhancement of the positive displacement effect arising from the increased instantaneous separation factor at the composition φ_0. In contrast, for mixtures of divergent solutes, a decrease in φ_0 is often detrimental owing to an decrease in the analytical separation and an enhancement of the negative tag-along effect arising from the decrease in the instantaneous separation factor at the composition φ_0.

For mixtures of convergent solutes, the solute elution order can be reversed during the gradient run (*i.e.*, the log k'_j *versus* φ plots cross each other inside the modifier concentration range of interest) and, in this instance, it is difficult to predict whether or not a preparative separation can be improved by increasing the initial mobile

phase composition. In order to follow the influence of φ_0 on such systems, the logarithmic mean separation factor α_{ln} defined by Antia and Horvath [5] can be used:

$$\alpha_{ln} = \frac{\alpha_{\varphi_0} - \alpha_{\varphi^*}}{\ln(\alpha_{\varphi_0}/\alpha_{\varphi^*})} \quad (10)$$

where α_{φ_0} is the separation factor for the initial mobile phase composition φ_0 and α_{φ^*} is the separation factor for the modifier volume fraction φ^* at which the capacity factor of the less retained solute k'_1 is unity (in practice, $\varphi_{f,1}$ and $\varphi_{f,2}$ are close to φ^*). The separation is expected to improve when α_{ln} increases.

4. OPTIMIZATION OF A PREPARATIVE GRADIENT ELUTION SEPARATION

The goal of a preparative liquid chromatographic operation is to recover, from a mixture, the largest amount of given components with specified requirement for the purity level and the recovery in the shortest time. Hence, the parameter to be optimized is the production rate P_R, which is the amount of selected component collected per run with the required specifications of purity and recovery, divided by the cycle time including any wash and regeneration steps. Both the recovered amount and the run time depend on the gradient conditions (gradient steepness G and initial mobile phase composition φ_0) and column plate number N (defined by the column length, particle diameter and mobile phase velocity). Hence there are some optimum values of column efficiency N_{opt}, gradient steepness G_{opt} and initial mobile phase composition $\varphi_{0,opt}$ for which the production rate is maximum. These optimum conditions were investigated from simulated runs [8]; the derived conclusions can be used as qualitative guidelines for the design of preparative gradient elution separations as follows.

For equimolar mixtures of parallel solutes, the production rate is roughly constant and maximum for gradient steepness values ranging from 0.15 to 0.3 ($0.15 < G_{opt} < 0.3$). The production rate is very weakly dependent on the initial mobile phase composition: initial mobile phase compositions leading to capacity factors ranging from 10 up to 100 give comparable production rates ($10 < k'_{0,j,opt} < 100$). The optimum column plate number N_{opt} which maximizes the production rate depends on both the retention of solutes and the required recovery; however, it was observed that the analytical resolution $R_{s,opt}$ corresponding to N_{opt} is only dependent on the recovery ratio. The gradient conditions having been optimized, the column conditions (mobile phase flow-rate for a laboratory-scale application) can be varied in order to obtain a certain analytical resolution: $R_{s,opt} \approx 1.6$ for a touching band separation, $R_{s,opt} \approx 1.2$ for 95% recovery of pure product and $R_{s,opt} \approx 0.9$ for 50% recovery of pure product.

For mixtures of convergent solutes, the decrease in the amount recovered with increasing gradient steepness or the increase in the amount recovered with decreasing initial mobile phase composition is faster than for mixtures of parallel solutes. Consequently, G_{opt} and $k'_{0,j,opt}$ for convergent solutes are smaller and higher, respectively, than for parallel solutes.

For mixtures of divergent solutes, the opposite is true: the amount recovered decreases more slowly than for parallel solutes when the gradient steepness is increased and G_{opt} for divergent solutes is larger than for parallel solutes. Finally, a strong initial retention of solutes can reduce dramatically the amount recovered and, consequently, $k'_{0,j,opt}$ for such a case is generally low.

5. CONCLUSIONS

Quantitative modelling of overloaded separations is more difficult for gradient than for isocratic elution. This implies fitting accurately competitive distribution isotherms over the whole useful range of mobile phase composition and, owing to the concentrating effect, over a wide range of solute concentration. However, the investigation of gradient elution under overload conditions by means of simplified simulation algorithms has not revealed any unexpected phenomena associated with column overloading and solute–solute interactions. It should simply be mentioned that the displacement and tag-along effects affect separations more significantly

than in isocratic elution. The selection of the optimum experimental conditions for preparative gradient elution separation requires the relative intensity of the two effects, which depends on both the type of the mixture to be separated and its composition, to be ascertained. Hence it is very important to measure the dependence of the capacity factor k'_j on the mobile phase composition φ $[S_j = -\mathrm{d}(\log k'_j)/\mathrm{d}\varphi]$ for the two adjacent compounds; the optimum conditions can change dramatically according to whether the solutes are parallel, convergent or divergent.

REFERENCES

1 R.E. Regnier, *Science*, 222 (1983) 245.
2 H. Colin, P. Hilaireau and M. Martin, *J. Chromatogr.*, 557 (1991) 137.
3 L.R. Snyder, G.B. Cox and P.E. Antle, *J. Chromatogr.*, 444 (1988) 303.
4 G.B. Cox, P.E. Antle and L.R. Snyder, *J. Chromatogr.*, 444 (1988) 325.
5 F.D. Antia and Cs. Horváth, *J. Chromatogr.*, 484 (1989) 1.
6 G.B. Cox, L.R. Snyder and J.W. Dolan, *J. Chromatogr.*, 484 (1989) 409.
7 L.R. Snyder, J.W. Dolan and G.B. Cox, *J. Chromatogr.*, 484 (1989) 437.
8 L.R. Snyder, J.W. Dolan and G.B. Cox, *J. Chromatogr.*, 540 (1991) 21.
9 G. Cretier, M. El Khabchi and J.L. Rocca, *J. Chromatogr.*, 596 (1992) 15.
10 S.G. Hu, D.D. Ho and M.M. Hossain, *J. Chromatogr.*, 605 (1992) 175.
11 I. Langmuir, *J. Am. Chem. Soc.*, 38 (1916) 2221.
12 A. Velayudhan and Cs. Horváth, *J. Chromatogr.*, 435 (1988) 221.
13 M.Z. El Fallah and G. Guiochon, *Anal. Chem.*, 63 (1991) 859.
14 M.Z. El Fallah and G. Guiochon, *Anal. Chem.*, 63 (1991) 2244.
15 L.R. Snyder, J.W. Dolan, D.C. Lommen and G.B. Cox, *J. Chromatogr.*, 484 (1989) 425.
16 G.B. Cox and L.R. Snyder, *J. Chromatogr.*, 590 (1992) 17.
17 G.B. Cox, *J. Chromatogr.*, 599 (1992) 195.
18 G. Cretier, L. Macherel, M. El Khabchi and J.L. Rocca, in M. Perrut (Editor), *Proceedings of the 9th International Symposium on Preparative and Industrial Chromatography, Nancy, April 6–8, 1992*, Institut National Polytechnique de Lorraine, Nancy, 1992, pp. 85–90.
19 L.R. Snyder, in Cs. Horváth (Editor), *High Performance Liquid Chromatography —Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, pp. 208–216.
20 M.A. Quarry, R.L. Grob and L.R. Snyder, *Anal. Chem.*, 58 (1986) 907.
21 S. Heinisch, J.L. Rocca and M. Feinberg, *J. Chemometr.*, 3 (1988) 127.
22 J.H. Knox and H.M. Pyper, *J. Chromatogr.*, 363 (1986) 1.
23 *Bioprep, User's Manual*, Medical Product Department, E.I. DuPont de Nemours, Wilmington, DE, 1989.
24 J.E. Eble, R.L. Grob, P.E. Antle, G.B. Cox and L.R. Snyder, *J. Chromatogr.*, 405 (1987) 31.
25 J. Newburger, L. Liebes, H. Colin and G. Guiochon, *Sep. Sci. Technol.*, 22 (1987) 1933.
26 G. Guiochon and S. Ghodbane, *J. Phys. Chem.*, 92 (1988) 3682.
27 J. Newburger and G. Guiochon, *J. Chromatogr.*, 484 (1989) 153.
28 S. Golshan-Shirazi and G. Guiochon, *J. Phys. Chem.*, 93 (1989) 4143.
29 A.M. Katti and G. Guiochon, *J. Chromatogr.*, 499 (1990) 21.
30 S. Golshan-Shirazi and G. Guicohon, *Anal. Chem.*, 62 (1990) 217.
31 S. Golshan-Shirazi and G. Guiochon, *Chromatographia*, 30 (1990) 613.
32 G. Cretier, L. Macherel and J.L. Rocca, *J. Chromatogr.*, 590 (1992) 175.
33 M. El Khabchi, *PhD Thesis*, University of Lyon, 1993.
34 A. Katti and G. Guiochon, *Anal. Chem.*, 61 (1989) 982.