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Preparative liquid chromatography

II. Existence of optimum injection conditions for overloaded gradient elution separations

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ABSTRACT

The effect of injection conditions on overloaded gradient elution separations in liquid chromatography was studied theoretically from simulated chromatograms. The simulation algorithm was based on the Craig machine. The separation of binary mixtures having both constant and non-constant separation factors with changing modulator concentration was examined. It appears that there is a general parallelism between gradient elution and isocratic elution under overload conditions: the injection concentration has to be optimized in order to maximize the recovered amount and the optimum injection conditions depend on the column efficiency.

INTRODUCTION

Gradient elution is widely used in preparative-scale liquid chromatography, especially to recover, in a single run, pure solutes from a complex mixture whose components exhibit a broad range of retentivity. In the gradient elution mode, the initial injection step is carried out under strong retention conditions, after which the eluent strength is increased continuously during the chromatographic run by progressive modification of the mobile phase composition. Because of the very low eluent strength of the initial mobile phase, immediately following sample injection, the solutes are retained in a very thin slice of the column inlet where their mobile phase concentration is very high, their distribution isotherm is very non-linear and the degree of interference between them is very strong. Consequently, the profile of solute bands at the column inlet is very distorted and, intuitively, decreasing the injected concentration could result in less band broadening and better resolution between two successive elution bands.

The influence of input concentration on the degree of separation has been studied in overloaded isocratic elution [1–4]. However, none of the recent attempts to model overloaded gradient elution separations for multi-component samples [3,5–12] dealt with this issue; all these studies, regardless of the role of injection conditions, assumed that only the magnitude of the sample load has to be taken into account.

The goal of preparative liquid chromatography is to recover, from a mixture, the largest amount of certain components with a specified purity, and in this work we investigated the effect of the sample concentration on the recovered amounts for binary mixtures of various compositions, chromatographed under linear-strength (LSS) gradient elution conditions [13,14] and having both constant and non-constant separation factors with changing mobile phase composition. The influence of column efficiency on the optimization of the injection conditions was also considered. This work is based on the use of our new approach using the Craig model,

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CRAIGSIM [4], that we first tested under analytical gradient conditions, *i.e.*, in gradient elution with a linear isotherm, in order to verify that it matches the LSS theory.

THEORY

Simulation

The CRAIGSIM software was detailed in Part I [4].

Elution conditions

In our simulation experiments, we consider only linear forms for the gradient profile and there is no system dwell volume, *i.e.*, the variation of the modifier concentration φ in the mobile phase at the column inlet against the eluted volume V is written as

$$\varphi = \varphi_0 \qquad V \leqslant V_i \tag{1}$$

$$\varphi = \varphi_0 + G(V - V_i) \qquad V > V_i \tag{2}$$

where φ_0 is the modifier concentration in the eluent before the start of the gradient run, V_i is the injection volume and G is the gradient steepness.

Characteristics of sample mixtures

In overloaded chromatography and for a two-component mixture, the equilibrium concentration of a solute j ($j = 1$ or 2) in the stationary phase depends on its own equilibrium concentration in the phase mobile and on the equilibrium mobile phase concentration of the other solute. Assuming a mixed Langmuir isotherm model for the solute distribution, the equilibrium concentrations of solutes 1 and 2 in the stationary phase, $C_{s,1}$ and $C_{s,2}$, and in the mobile phase, $C_{m,1}$ and $C_{m,2}$, are related by

$$C_{s,1} = \frac{a_1 C_{m,1}}{1 + b_1 C_{m,1} + b_2 C_{m,2}} \tag{3}$$

$$C_{s,2} = \frac{a_2 C_{m,2}}{1 + b_2 C_{m,2} + b_1 C_{m,1}} \tag{4}$$

where the isotherm coefficients a_j and b_j ($j = 1$ or 2) depend on the mobile phase composition φ. a_j is related to the isocratic capacity factor, k'_j, of the solute by means of the total porosity ε of the column packing according to $a_j = \varepsilon k'_j/(1 - \varepsilon)$ and, in reversed-phase chromatography, the variation of a_j with φ can usually be described by the empirical relationship

$$a_j = a_{0,j} \cdot 10^{-m_j \varphi} \tag{5}$$

where $a_{0,j}$ is the a_j value for pure water as mobile phase and m_j is a constant that depends on both the solute and the organic modifier used in the organic–water mobile phase. On the assumption that the saturation concentration of the stationary phase, a_j/b_j, is a constant $C_{s,0}$ independent of both the solute and the mobile phase composition, the isotherm coefficient of non-linearity, b_j, is given by

$$b_j = \frac{a_{0,j}}{C_{s,0}} \cdot 10^{-m_j \varphi} \tag{6}$$

Fig. 1 shows the three different types of sample mixture that can be distinguished. For mixture type A, the m_1 and m_2 values are equal, the $\log a_j$ *versus* φ plots are parallel (Fig. 1a) and, consequently, the separation factor defined by

$$\frac{a_2}{a_1} = \frac{a_{0,2}}{a_{0,1}} \cdot 10^{-(m_2 - m_1)\varphi} \tag{7}$$

is kept constant as the mobile phase composition φ is changing. For the two other mixture types, m_1 and m_2 differ and the separation factor a_2/a_1 is non-constant while changing the modifier concentration. For mixture type B ($m_1 > m_2$) the $\log a_i$ *versus* φ plots are divergent and a_2/a_1 increases with increase in φ (Fig. 1b). For mixture type C ($m_1 < m_2$), the $\log a_i$ *versus* φ plots are convergent and a_2/a_1 decreases with increase in φ (Fig. 1c). As suggested by Antia and Horváth [11], the three mixture types A, B and C are denoted parllel, divergent and convergent solutes, respectively.

LSS theory

According to the linear solvent strength theory [13], under analytical gradient conditions, the retention volume $V_{R,j}$ of a component j and the standard deviation expressed in volume units, σ_j, of its peak are given by

$$V_{R,j} = V_M + \frac{1}{m_j G} \cdot \log[1 + 2.3 m_j G V_M \cdot \frac{(1 - \varepsilon)}{\varepsilon} \cdot a_{0,j} \cdot 10^{-m_j \varphi_0}] \tag{8}$$

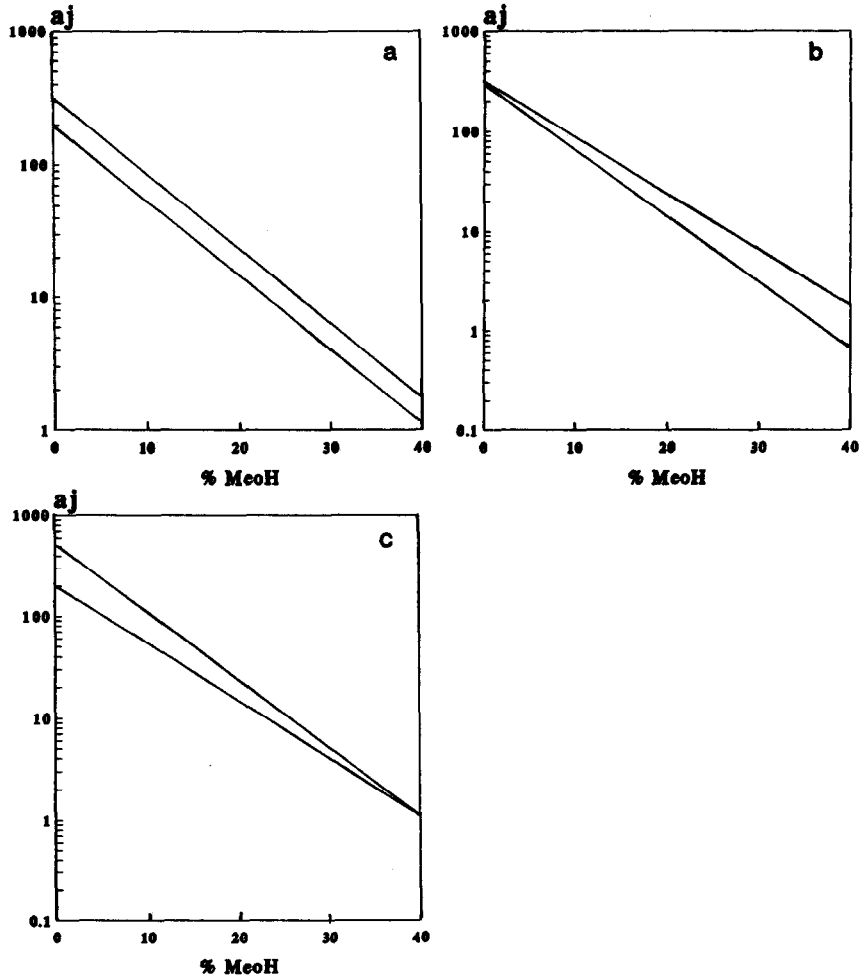

Fig. 1. Log a_j *vs.* φ plots corresponding to the various mixture types (see Table I). (a) Mixture type A (parallel solutes); (b) mixture type B (divergent solutes); (c) mixture type C (convergent solutes).

and

$$\sigma_j = \frac{gV_M}{\sqrt{N_j}}\left(1 + \frac{\frac{1-\varepsilon}{\varepsilon}\cdot a_{0,j}\cdot 10^{-m_j\varphi_0}}{1 + 2.3m_jGV_M\cdot\frac{1-\varepsilon}{\varepsilon}\cdot a_{0,j}\cdot 10^{-m_j\varphi_0}}\right) \tag{9}$$

where V_M is the column dead volume, g is the band compression factor defined by

$$g = \frac{\sqrt{1 + p + p^{2/3}}}{1 + p} \tag{10}$$

with

$$p = \frac{2.3m_jGV_M\cdot\frac{1-\varepsilon}{\varepsilon}\cdot a_{0,j}\cdot 10^{-m_j\varphi_0}}{1 + \frac{1-\varepsilon}{\varepsilon}\cdot a_{0,j}\cdot 10^{-m_j\varphi_0}} \tag{11}$$

and N_j is the column plate number. N_j can be measured under isocratic conditions selected to be equivalent to those in gradient elution (see discussion in ref. 14), *i.e.*, under isocratic conditions for which the capacity factor k'_j is equal to the median capacity factor during the gradient run $\overline{k'_j}$ (value of

the capacity factor when the band has migrated half way along the column). $\overline{k'_j}$ is given by

$$\overline{k'_j} = \frac{\frac{1-\varepsilon}{\varepsilon} \cdot a_{0,j} \cdot 10^{-m_j \varphi_0}}{1 + 1.15 m_j G V_M \cdot \frac{1-\varepsilon}{\varepsilon} \cdot a_{0,j} \cdot 10^{-m_j \varphi_0}} \quad (12)$$

In a simulated isocratic separation, the column plate number N_j is equal to $(n_c + 1)[(1 + k'_j)/k'_j]$, where n_c is the stage number of the Craig machine [15]. Similarly, in a simulated gradient run, N_j is assumed to be related to n_c by the following equation [12]

$$N_j = (n_c + 1)\frac{1 + \overline{k'_j}}{\overline{k'_j}} \quad (13)$$

Reduced production rate

The production rate of solute j, $R_{H,j}$, is defined as amount of solute j recovered with the required purity level, $Q_{r,j}$, divided by the run time, T:

$$R_{H,j} = \frac{Q_{r,j}}{T} \quad (14)$$

The run time in our study extends from the start of the injection to the point where the last traces of the second component elute:

$$T = \frac{V_{E,2}}{D} \quad (15)$$

where D is the elution flow rate and $V_{E,2}$ is the elution volume when the eluted concentration of solute 2 is equal to 10^{-8} mol/l.

We define the reduced production rate of solute j, $r_{H,j}$, as production rate of the solute j, $R_{H,j}$, divided by the elution flow rate, D. Substitution of eqn. 15 in eqn. 14 gives

$$r_{H,j} = \frac{R_{H,j}}{D} = \frac{Q_{r,j}}{V_{E,2}} \quad (16)$$

EXPERIMENTAL

Equipment, materials and procedures were the same as in Part I [4]. The simulated column was 150 mm × 5 mm I.D. with a total porosity ε of 0.8; hence its dead volume V_M was 2.36 ml. The column plate number N_j was varied by changing the number of stages n_c in the Craig machine.

The gradient profile simulated corresponded to $\varphi_0 = 0$ and $G = 0.015$ ml^{-1}. Table I summarizes the retention characteristics of the various sample mixtures studied. The analytical resolution is identical for each of these three samples owing to the equal values of $(V_{R,j} - V_M)/V_M$ for $j = 1$ and $j = 2$, respectively. Plots of log a_j *versus* φ are shown in Fig. 1.

RESULTS AND DISCUSSION

Comparison between CRAIGSIM results and LSS theory for analytical gradient separations

Fig. 2 shows the chromatograms of the sample mixture of type A at a relative composition of 1:1, obtained under analytical injection conditions and simulated for stage numbers of 200 and 600. The accuracy of the CRAIGSIM algorithm previously demonstrated in preparative isocratic elution [4] is

TABLE I

CHARACTERISTICS OF THE VARIOUS MIXTURE TYPES CONSIDERED

Mixture type	Solute	$a_{0,j}$	m_j	$C_{s,0}$ (mol/l)	$\frac{V_{R,j} - V_M}{V_M}$	$\overline{k'_j}$
A (parallel solutes)	1	200	5.65	10	6.90	4.00
	2	320	5.65		7.89	4.12
B (divergent solutes)	1	300	6.65	10	6.88	3.53
	2	320	5.65		7.89	4.12
C (convergent solutes)	1	200	5.65	10	6.90	4.00
	2	520	6.65		7.88	3.60

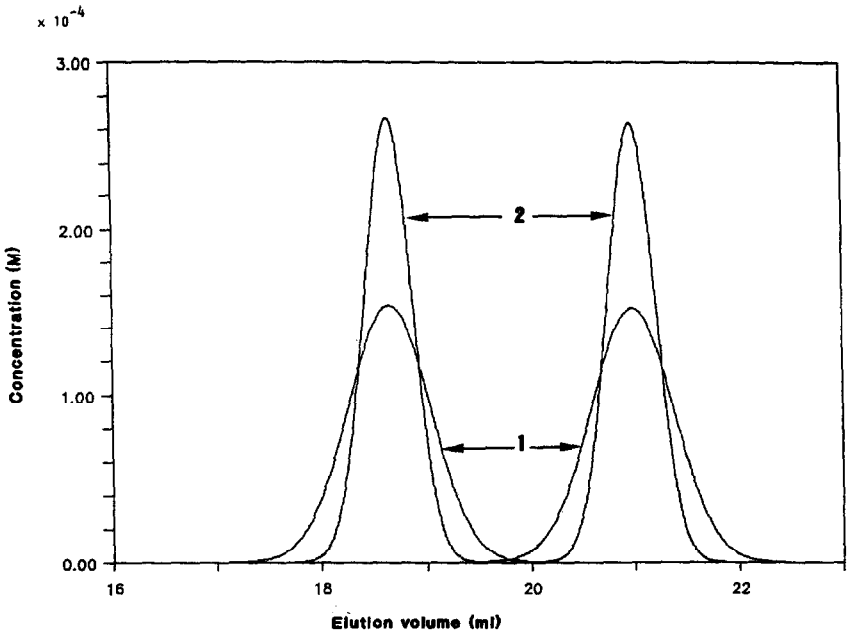

Fig. 2. Analytical chromatograms of the sample mixture of type A. Relative mixture composition: 1:1. Injection conditions: $C_i = 2.5 \cdot 10^{-2}$ mol/l; $Q_i = 3 \cdot 10^{-7}$ mol. Elution conditions: $\varphi_0 = 0$; $G = 0.015\,\mathrm{ml}^{-1}$. Curves: (1) $n_c = 200$ ($N_1 = 251$, $N_2 = 250$); (2) $n_c = 600$ ($N_1 = 751$, $N_2 = 747$).

confirmed in analytical gradient elution by comparing the values of the retention volumes and the peak standard deviations measured from simulated chromatograms in Fig. 2 with those calculated from the LSS theory, eqns. 8 and 9 (Table II). The values measured from CRAIGSIM simulations match those predicted by the LSS theory, within 0.05% for the retention volume and 1.5% for the peak standard deviation.

TABLE II

COMPARISON OF THE RETENTION VOLUMES AND PEAK STANDARD DEVIATIONS MEASURED FROM CRAIGSIM SIMULATIONS AND CALCULATED FROM LSS THEORY EQUATIONS

Mixture type: parallel solutes (A). Mixture composition: 1:1.

Solute	n_c	N_j[a]	$V_{R,j}$ (ml)		σ_j (ml)	
			Measured[b]	Calculated[c]	Measured[d]	Calculated[e]
1	200	251	18.65	18.65	0.395	0.390
	600	751	18.65	19.65	0.226	0.225
2	200	250	20.98	20.97	0.401	0.395
	600	747	20.97	20.97	0.229	0.228

[a] Calculated by eqn. 13.
[b] Elution volume of the peak maximum on the simulated chromatogram.
[c] Value calculated by eqn. 8.
[d] Half-width of the band measured at 0.607 of the peak height on the simulated chromatogram.
[e] Value calculated by eqn. 9.

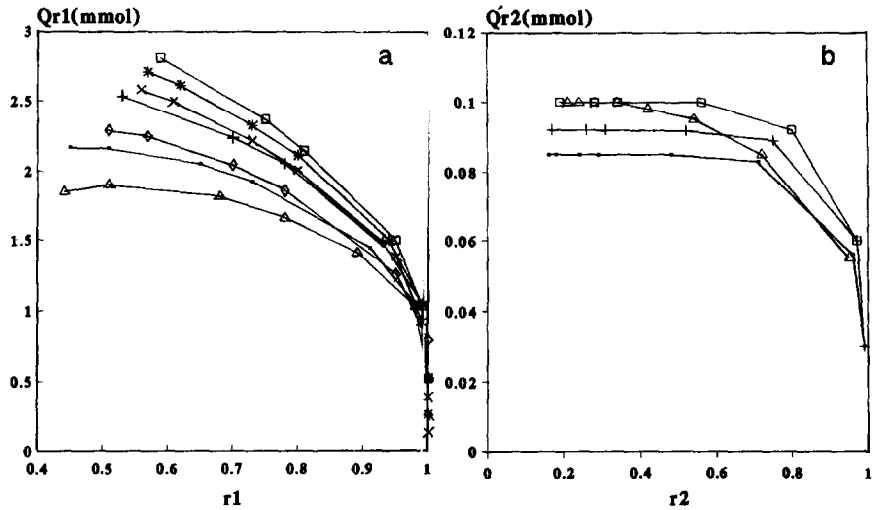

Fig. 3. Plot of the recovered amount at 99% purity *versus* the recovery yield for different sample concentrations. Mixture: relative composition = 9:1, type A (see Table I). $n_c = 200$ ($N_1 = 251$, $N_2 = 250$). C_i: ■ = 0.1; + = 0.2; ✳ = 0.4; □ = 1.7; × = 12.5; ◇ = 25; △ = 50 mol/l. (a) Component 1; (b) component 2.

Optimization of sample size and sample concentration for preparative gradient runs

The effect of the sample load Q_i and the sample concentration C_i on the recovery ratios, r_1 and r_2, and the recovered amounts, $Q_{r,1}$ and $Q_{r,2}$, of components 1 and 2, respectively, at 99% purity was studied for three different relative compositions, 9:1, 1:1 and 1:9, of the three binary sample mixtures mentioned in Table I. The results are qualitatively similar whatever the sample composition and we have only reported the results obtained with the 9:1 mixtures, for which the trends are much more pronounced.

Mixture of parallel solutes. Fig. 3 illustrates the effect of increasing sample size at constant injected concentration and plots the evolution of the re-

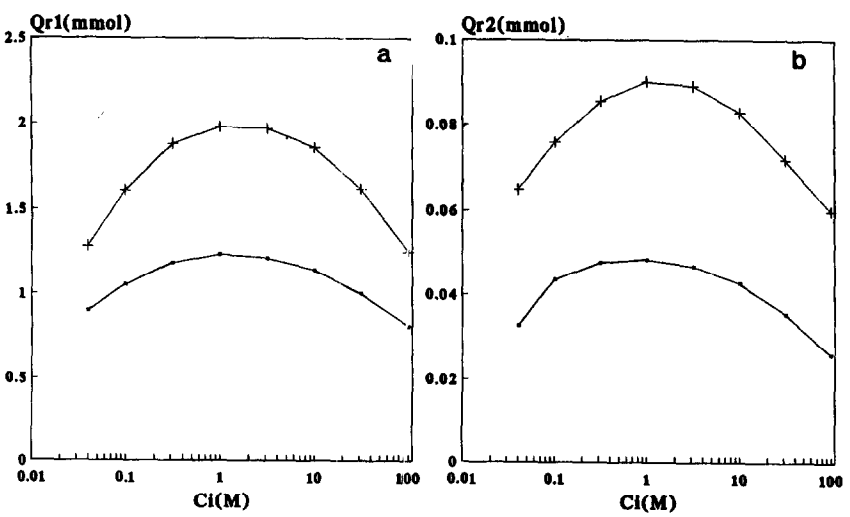

Fig. 4. Plot of the recovered amount at 99% purity *versus* the sample concentration for different recovery ratios. Mixture: relative composition = 9:1, type A (see Table I). $n_c = 200$ ($N_1 = 251$, $N_2 = 250$). r_j: (■) 98%; (+) 85%. (a) Component 1; (b) Component 2.

covered amount at 99% purity $Q_{r,j}$ *versus* the recovery yield r_j for different sample concentrations C_i. By increasing the sample amount, the chromatogram changes from Gaussian peaks (under analytical conditions) to touching bands and, finally, to overlapping bands. The recovery ratio remains nearly constant and equal to 100% for all sample concentrations up to a certain injected amount. Under these conditions, the band overlap is small and all the injected solute amounts can be recovered with 99% purity. Beyond this point, the bands overlap too much, the recovery of the whole injected amount is no longer possible and the recovery ratio falls more or less rapidly, depending on both the sample concentration injected and the solute considered.

The study of the variation of the recovered solute amount against the injected sample concentration for a specified solute recovery ratio shows that there is an optimum sample concentration giving the highest recovered amount (Fig. 4). For solute 1, this optimum is more and more critically defined with decreasing recovery ratio. In contrast, for solute 2, the maximum of the recovered amount is sharper with increasing recovery ratio. However, for each solute, the optimum injection concentration seems to be independent of the fixed recovery ratio. Fig. 4 also illustrates that, in preparative chromatography, the fact of allowing as certain loss of product is very advantageous: it is possible to recover about 1.5–2 times as much 99% pure solute with a recovery of 85% as with a recovery of 98%.

The influence of the column efficiency on the optimum injection conditions is shown in Fig. 5, which plots, for different column plate numbers, the variation of the recovered amount $Q_{r,j}$ of 99% pure solute 1 against both the sample concentration C_i and the reduced injection volume V_i/σ_1 (ratio of the injection volume to the standard deviation of the Gaussian peak observed at infinite dilution). The larger the column plate number, the higher is the recovered amount and the more concentrated the sample has to be injected to recover the maximum amount. In contrast, the optimum value of the reduced injection volume seems not to be affected by the column efficiency.

All the preceding observations parallel those found for the case of isocratic overlapping band separation [4]. This confirms the essential similarity of the overloaded separations in isocratic or gradient elution, which was mentioned previously [8,10,12]. Figs. 6 and 7 also demonstrate that the phenomena explaining the existence of optimum injection conditions in gradient elution are the same as in isocratic elution. Fig. 6 superposes the band profiles corresponding to injection of the 9:1 mixture

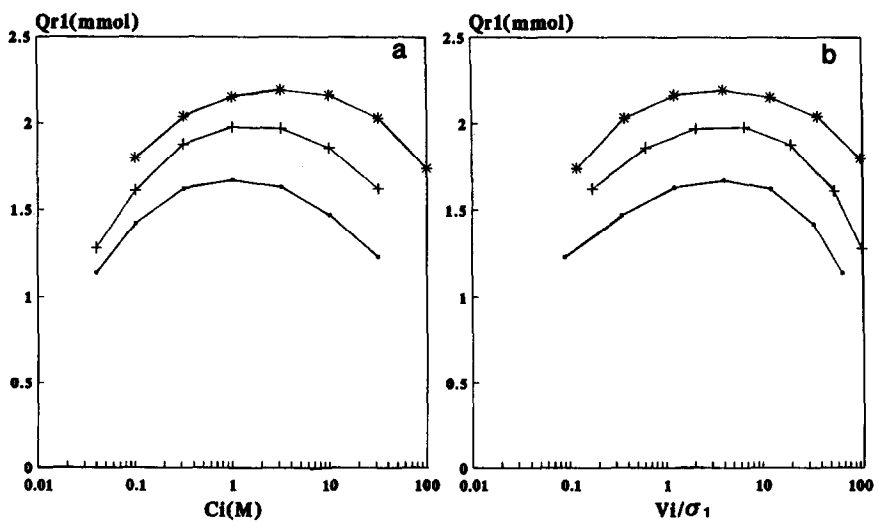

Fig. 5. Plot of the recovered amount of 99% pure solute 1 *versus* (a) the sample concentration and (b) the reduced injection volume for different column efficiencies. Mixture: relative composition = 9:1, type A (see Table I). r_1 = 85%. (■) n_c = 100 (N_1 = 126, N_2 = 126); (+) n_c = 200 (N_1 = 251, N_2 = 250); (∗): n_c = 600 (N_1 = 751, N_2 = 747).

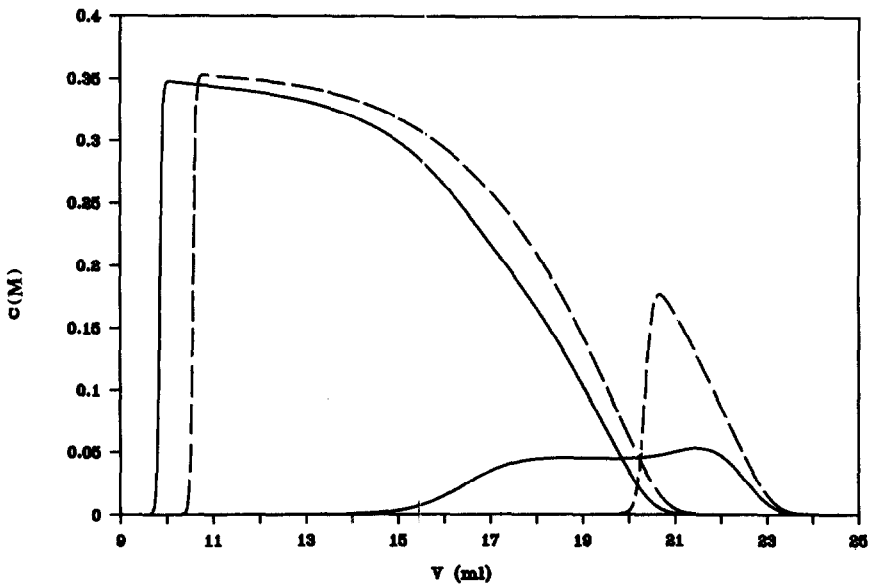

Fig. 6. Chromatograms obtained by (solid line) direct injection of the mixture and (dashed line) superposition of the band profiles corresponding to the equivalent amounts of single solutes. Q_i = 2.96 mmol; C_i = 1.7 mol/l. Mixture: relative composition = 9:1, type A (see Table I). n_c = 200 (N_1 = 251, N_2 = 250).

A (full line) and to injection of the equivalent amounts of single solutes (dashed line). Clearly, there are displacement and tag-along effects for overload separations in the gradient elution mode, as already seen in the isocratic elution mode (compare with Fig. 2 in ref. 4). However, in contrast to the right triangle appearance of the isocratic bands, the rounded shape of the more heavily overloaded band (solute 1) reflects the fact that the band tail in gradient elution always travels in a stronger mobile phase. Fig. 7 compares three chromatograms corresponding to the same amount injected at three different concentrations (this figure should be compared with Figs. 11 and 12 in Part I [4]). The injected sample amount was kept constant at 2.96 mmol. The elution profiles 1, 2 and 3 were obtained for injected concentrations of 0.1, 1.7 and 27.8 mol/l, respectively. The corresponding recovery ratios of 99% pure first component are 72.6, 81.0 and 72.3%. Hence the elution profile 2, which corresponds to the optimum injection concentration for the recovery of the first-eluted solute, results from a compromise between two simultneous phenomena: when the injected concentration is increased and the injected volume is decreased (the injected amount being constant), classically the peak width is decreased (comparison of elution profiles 1 and 2) and consequently the first component recovery ratio is increased; however, for a high injection concentration, owing to the tag-along effect, the front base of the second component peak is attracted under the first component peak (comparison of elution profiles 2 and 3). This additional band broadening is responsible for the decrease in the first component recovery ratio for high injected concentrations. In contrast to isocratic elution (Figs. 11 and 12 in ref. 4), the rear portion of the second component which does not overlap with the first component does not exhibit a plateau, but forms a hump because the compressive effect of the gradient acts to concentrate it.

Like the recovered amount, the production rate for given conditions of column, flow-rate, recovery ratio and purity (*i.e.*, the reduced production rate) also depends on the injection concentration. Fig. 8 shows that both recovered amount and reduced production rate are maximum for certain values of the sample concentration, but the optimum sample concentration for the reduced production rate is larger than that for the recovered amount. The

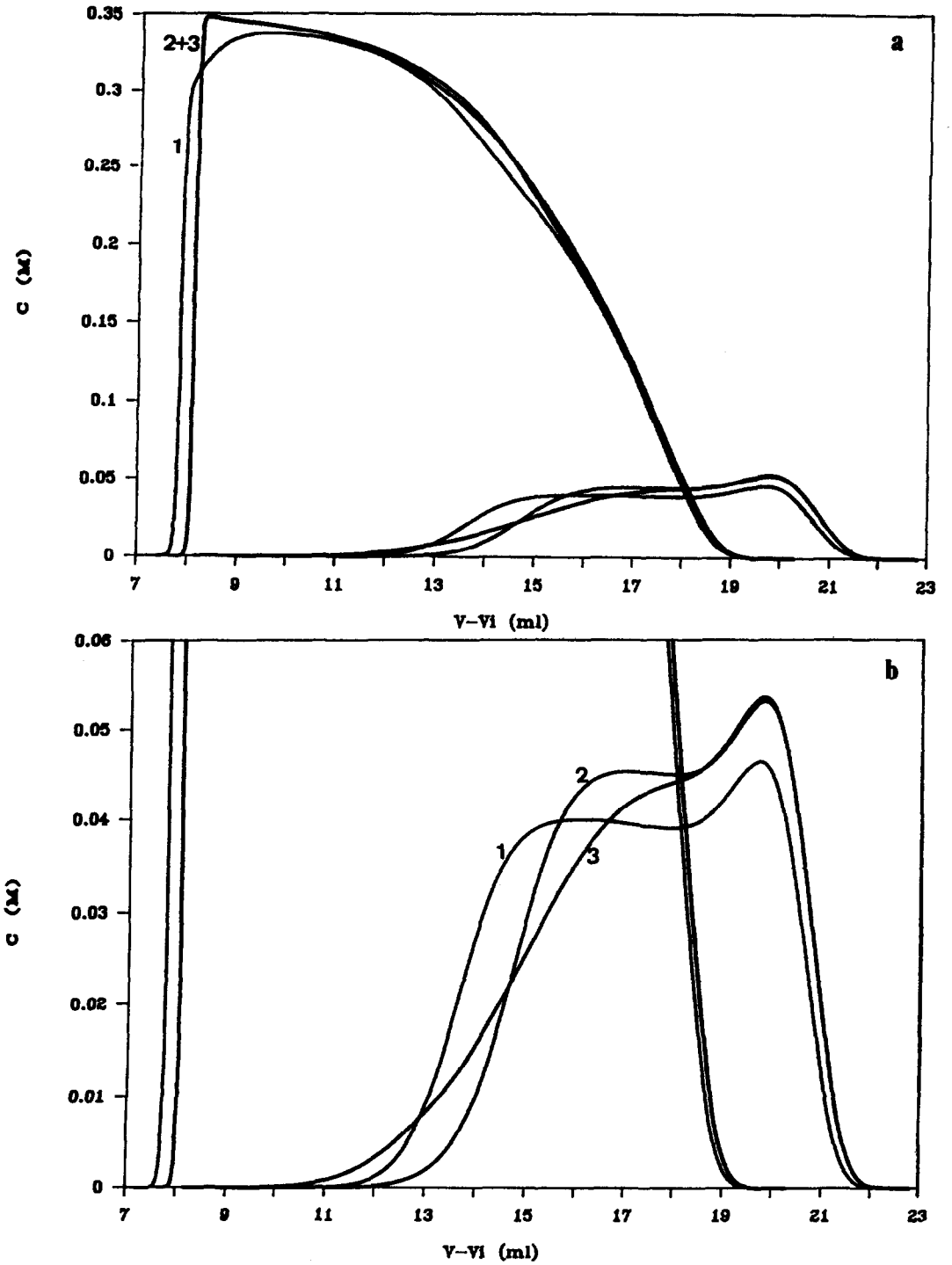

Fig. 7. Three chromatograms corresponding to the same injected amount, Q_i = 2.96 mmol. Mixture: relative composition = 9:1, type A (see Table I). n_c = 200 (N_1 = 251, N_2 = 250). (1) C_i = 0.1 mol/l; (2) C_i = 1.7 mol/l; (3) C_i = 27.8 mol/l (the elution volume is adjusted by subtracting the injection volume). (a) Global chromatograms: (b) enlargement of the seconds peaks.

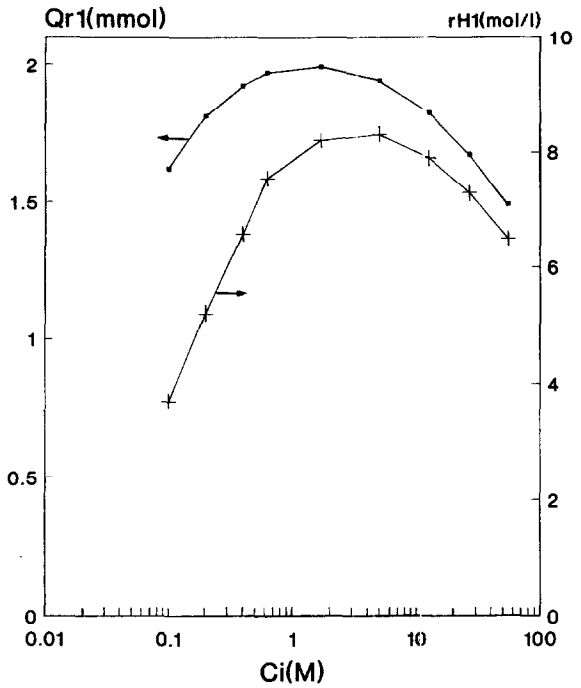

Fig. 8. Dependence of the recovered amount and reduced production rate for 99% pure solute 1 as a function of sample concentration. Mixture: relative composition = 9:1, type A (see Table I). r_i = 85%. n_c = 200 (N_1 = 251, N_2 = 250).

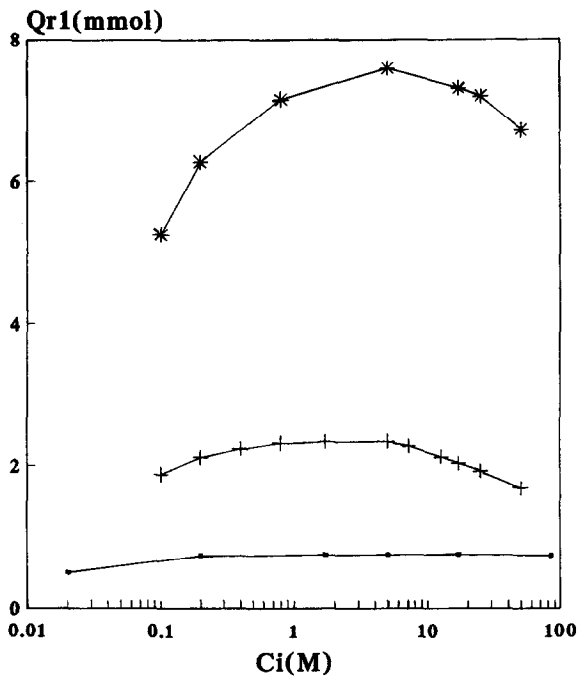

Fig. 9. Plot of the recovered amount of 99% pure solte 1 *versus* the sample concentration for different mixture types. Relative mixture composition: 9:1. r_1 = 75%. n_c = 200 (N_1 = 251, N_2 = 250). + = Mixture type A (parallel solutes): ■ = mixture type B (divergent solutes); ✳ = mixture type C (convergent solutes).

reason is that the run time varies as a function of injection concentration: when the injection concentration is decreased, the injected volume is increased; hence the feed time, the delay before the gradient is introduced at the column inlet and consequently the run-time are increased.

Mixtures with non-constant separation factor. Comparison of the behaviours of parallel, divergent and convergent solute pairs having the same resolution in analytical gradient elution is shown in Fig. 9, which plots the first component amount recovered at 99% purity with a yield of 75% against the sample injection concentration for each mixture type (see Table I). As demonstrated by Snyder *et al.* [10,12], the maximum recovered amount and, consequently, the injectable sample load are increased when we successively consider mixtures of divergent, parallel and convergent solutes. Fig. 9 also shows that the optimum injection concentration is more critically defined for convergent solutes (compared with parallel solutes) and much less marked for divergent solutes. This results from two opposite effects. On the one hand, the injection volume corresponding to the beginning of the column volume overload is independent of the sample size and the corresponding injection concentration decreases with decreasing sample size. On the other hand, as the sample size decreases, the tag-along effect becomes weaker [16] and band broadening of the front base of the second-eluted component appears for higher injected concentrations. This explains why the recovered amount from divergent solute mixtures remains nearly constant within a broad range of injected concentrations.

CONCLUSIONS

We have studied gradient elution under overload conditions for binary mixtures. The findings parallel those deduced from overloaded isocratic elution. For given preparative specifications of recovery ratio and purity level, the maximum amount re-

covered is obtained for certain injection conditions. These optimum injection conditions are critically defined for mixtures of convergent solutes. These cases also correspond to larger samples that can be separated and it is important to optimize accurately the injection conditions with such mixtures. The column efficiency also influences the optimum injection conditions. The optimum injection volume corresponding to the maximum amount recovered is directly proportional to the standard deviation of the Gaussian peak observed at infinite dilution.

SYMBOLS

a_j initial slope of the Langmuir isotherm of solute j (j = 1 or 2)
$a_{0,j}$ value of a_j for $\varphi = 0$ (eqn. 5)
b_j coefficient of non-linearity of Langmuir isotherm of solute j (j = 1 or 2)
C_i sample concentration
$C_{m,j}$ equilibrium concentration of solute j in mobile phase (j = 1 or 2)
$C_{s,0}$ value of the saturation concentration of the stationary phase for solute 1 or 2 and whatever the mobile phase composition
$C_{s,j}$ equilibrium concentration of solute j in stationary phase (j = 1 or 2)
D elution flow-rate
g band compression factor (eqn. 10)
G linear rate of change of modulator concentration with elution volume
k'_j capacity factor of solute j under isocratic conditions (j = 1 or 2)
$\overline{k'_j}$ median capacity factor during the gradient run (eqn. 12)
m_j parameter that measures change in a_j value with change in φ (eqn. 5)
n_c stage number of the Craig machine
N_j column plate number under corresponding isocratic conditions
p parameter defined by eqn. 11
Q_i sample amount
$Q_{r,j}$ recovered amount of solute j at a certain purity (j = 1 or 2)
$r_{H,j}$ reduced production rate of solute j (j = 1 or 2) (eqn. 16)
r_j recovery ratio of solute j (j = 1 or 2)
$R_{H,j}$ production rate of solute j (j = 1 or 2)
T run time
V elution volume
$V_{E,2}$ end-point of the chromatogram; elution volume when the eluted concentration of solute 2 is equal to 10^{-8} mol/l
V_i injected volume of sample
V_M column dead volume
$V_{R,j}$ retention volume of solute j under analytical gradient conditions (j = 1 or 2)
ε total porosity of the chromatographic bed
φ modifier concentration in the mobile phase
φ_0 modifier concentration at the start of the gradient
σ_j standard deviation expressed in volume units of the Gaussian peak observed under analytical gradient conditions (j = 1 or 2)

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