Journal of Chromatography, 535 (1990) 93–110
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2042

Studies on system peaks in ion-pair adsorption chromatography

IV. Optimization of peak compression

T. FORNSTEDT and D. WESTERLUND*
Department of Analytical Pharmaceutical Chemistry, Biomedical Centre, Uppsala University, P.O. Box 574, S-751 23 Uppsala (Sweden)
and
A. SOKOLOWSKI
Pharmacia LKB Biotechnology, Chemical Production, 751 82 Uppsala (Sweden)

ABSTRACT

Optimization of a chromatographic system in order to obtain extremely narrow analyte peaks was investigated. The system consisted of silanized silica as a solid phase and an acidic eluent containing an UV-detectable hydrophobic amine (the probe). Large probe deficiency peaks (system peaks) were generated by injecting high concentrations of an organic anion together with the analyte. In the rear part of the large system peaks the probe concentration increased steeply. Strong peak compression effects were obtained when small amounts of cationic analytes were eluted together with this co-ion gradient. Variation of the probe concentration in the eluent was an efficient way of obtaining peak matching. Peak deformations developed at high analyte concentrations and/or when the analyte was eluted in other parts of the system peak. Guidelines for increasing the peak compression effect and to avoid the deformations are presented.

INTRODUCTION

Possibilities of obtaining narrow chromatographic peaks are of special interest owing to the accompanying improvement of the detection limit. In gradient elution this can be achieved by a continuous increase in the eluent component with the greatest eluting strength [1]. This gives a larger increase in the migration speed of the rear part of the analyte zone, resulting in narrower peaks than in corresponding isocratic runs.

In reversed-phase ion-pair adsorption chromatography, gradients of organic ionic components can be utilized in order to obtain extremely narrow peaks. This was achieved for cationic analytes by a stepwise increase in the eluent concentration of an organic co-ion (an ion with the same charge as the analyte) [2–4]. The drawback of the technique is the long re-equilibration times required. A technique offering extremely strong peak compression effects of cationic analytes with relatively short run times involves the utilization of large system peaks of an organic co-ion present in the eluent [5–7].

0021-9673/90/$03.50

System peaks, in which the eluent components have concentration deviations from the bulk, are generated by the injection of a sample deviating in composition from the eluent [8]. If the system is equilibrated with an organic cation in the eluent, large negative system peaks containing a deficiency of the eluent cation can be induced by the injection of high concentrations of a hydrophobic organic anion. Large peak compression effects were then obtained when the simultaneously injected cationic analyte was eluted together with the steeply increasing co-ion (cation) concentration in the rear part of the system peak [5–7]. When the analyte was eluted in other positions within the system peak, peak deformations were developed [5,9]. Deformations could also be developed when the analyte was eluted in the position normally giving peak compression [5], provided that high analyte concentrations were injected and/or that the simultaneously injected organic anion was eluted close to the analyte. Distorted peak performances, such as peak deformation or even peak splitting [9–15], have been reported in many instances when eluents including an ionic organic component were used. In most explanations for these effects, system peak interactions with the analytes were, however, not considered [11–16].

The peak compression and deformation effects and the parameters governing the retention volume of the co-ion gradient and the analyte have been investigated earlier [5,7,17]. Rough matching of gradient and analyte retention volumes, in order to achieve co-elution, was obtained by varying the co-ion eluent concentration [7]. Fine matching could be made by changing the anion concentration in the injected solution [5].

The aims of this optimization study were twofold: to increase the degree of the peak compression effect and to avoid the risk of peak deformation occurring when designing the system for peak compression. In addition, some aspects of the selectivity of the technique are discussed below.

EXPERIMENTAL

Apparatus, chromatographic technique and preparation of the eluent

These were as described previously [17,18].

Eluents with four different protriptyline (PT) concentrations were equilibrated with the solid phase Nucleosil C_{18}, 5 μm [7]. The ionic strength ($I = 0.05$) and pH (2.0) were kept constant. For all injections a 100-μl loop was used.

Chemicals

These were the same as used previously [7].

Detection technique

For eluents lacking the probe (PT), the analyte signal was measured at 235 nm. The organic anion was then registered with a Beckman 156 refractive index (RI) detector.

When the eluent contained the probe, the photodiode-array UV detector used allowed parallel signal recording of the analyte and the probe. For a sample including tricyclic amines, a compensating detection technique was utilized to register the analyte signal [5,7,17,18]. This technique could not be used at high probe concentrations owing to baseline interferences in the analyte signals due to incomplete

compensation. Under these conditions the sample only contained benzamides. The analyte signal was then registered at 350 nm, where the probe had no absorbance. The probe signal was measured at 323 nm at the two lowest probe concentrations, whereas longer wavelengths were chosen for higher probe concentrations [7], except in the experiments presented in Figs. 7 and 8b.

Separation terms

A zone is defined as a region in the column where the composition of the mobile phase deviates from the eluent. The injection zone or starting zone is then the initial part of the column in which the component to be separated will be located, before the migration along the column. A peak is the portion of the chromatogram which corresponds to the eluted and detected zone. An exception is the very large deficiency zones of the probe, which lacked a sharp region of minimum concentration. These zones were named "zones" also when they appeared on the chromatogram (see Fig. 1).

The analyte peak retention volume is measured at the peak maximum (Fig. 1). The peak width, w_b (ml), is measured as the distance between the inflection points of the two peak tangents and the baseline. The depth, ΔC, of the negative system zone or peak is measured as the distance between the baseline and the zone minimum, given in concentration units (M). The width of the probe gradient in the rear part of the zone, ΔV, is determined according to Fig. 1 and given in volume units (ml). The gradient steepness, obtained by taking the ratio $\Delta C/\Delta V$, is then given as molarity per millilitre (M/ml). The gradient retention volume, $V_{R,G}$, is measured at half the upslope of the probe gradient.

RESULTS AND DISCUSSION

Solid phases such as silanized silica have often been found to contain more than one kind of adsorption site [17–20]. A previous adsorption study of PT as the phosphate ion-pair to Nucleosil C_{18} indicated a two-site adsorption behaviour of the

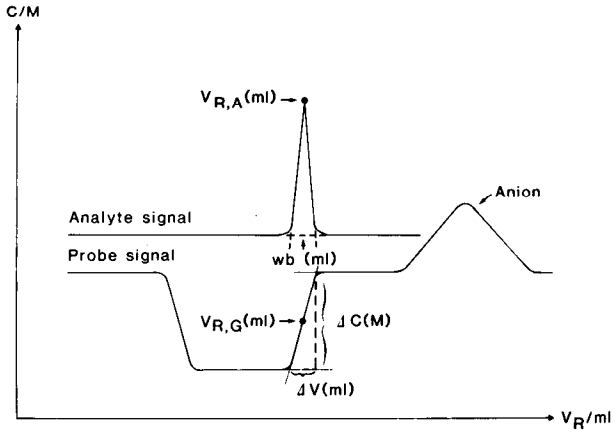

Fig. 1. Schematic presentation of the peak compression situation. The large probe system zone is induced by injection of an anion with a larger retention than the probe. The simultaneously injected analyte was eluted together with the probe gradient.

Langmuir type [17]. It was assumed that the adsorption isotherm is non-linear when more than 10% coverage of the adsorption capacity is reached [17–21]. In this series of studies, the primary variable was the eluent concentration of the probe, PT. The two highest probe concentrations corresponded to the non-linear part of the adsorption isotherm. At the highest probe concentration, the strong site was covered to the extent of 78%. The cationic analytes were substituted benzamides [17] and desipramine, imipramine and nortriptyline [19].

The retention equations used are based on the stoichiometric ion-pair adsorption model [7,8,17–20]. Eqn. 1 describes the net retention volume of a cationic analyte, HA^+, in the presence of an organic cationic eluent component, the co-ion Q^+; X^- is a buffer component. To simplify the equation, the expression for a homogeneous surface is used.

$$V_{N,HA} = \frac{W_s K_0 K_{HAX}[X^-]_m}{1 + K_{QX}[Q^+]_m[X^-]_m} \quad (1)$$

The total adsorption capacity of the solid phase is given by K_0, and K_{HAX} and K_{QX} are the adsorption constants. This equation assumes symmetrical peaks, whereas for high analyte concentrations no quantitative equation is yet available. Similar equations are valid for the net retention volumes of the organic anion injected and for the system peak [7,8,17–20].

At high sample concentrations there are interactions between all zones at the column inlet (the starting zones), and certainly also between unresolved zones during the migration along the column before the separation. When injecting cations and anions simultaneously, this will lead to retention changes in comparison with single-component injections, even though the components are well separated at the column outlet. In the retention equation discussed, these interactions are not taken into consideration.

Retention regulation

The retention regulation of the system has been described earlier [5,7,17]. In line with eqn. 1, the retention volume of the cationic analyte decreased with increasing PT (co-ion) concentration. If the cationic analyte was injected together with a large amount of organic anion, the analyte retention volume increased [5], an effect which was more pronounced when the analyte and the anion had similar retention volumes. The retention volume of the organic anion increased with increasing PT (counter ion) concentration.

As a consequense of the non-linear adsorption behaviour, the probe system peak retention volume decreased with increasing probe concentration. In order to obtain negative probe system peaks, the organic anion injected must have a larger retention volume than the probe; consequently, less hydrophobic anions could be used with higher probe concentrations [7] to achieve this.

Peak compression

Conditions necessary to obtain peak compression have been described earlier [5,7]. Large negative system peaks are generated by the injection of high concentrations

of alkylsulphonate or -sulphate with higher retention volumes than the probe. The probe concentration in such a probe zone is very low, whereas the rear part of the zone consists of a steeply increasing probe gradient (Fig. 1). An estimate of the degree of probe equilibrium disturbance is the ratio between the zone depth and the bulk concentration of the probe, $\Delta C/C_b$ [7]. This ratio is close to 1.0 at very large equilibrium disturbances. As described earlier, peak compression is achieved when the simultaneously injected cationic analyte is eluted together with the increasing co-ion gradient in the rear part of the zone (*cf.*, Fig. 1). The degree of the peak compression effect was measured as the decrease in peak width (w_b) compared with the isocratic experiment. Optimum matching of the analyte peak and co-ion gradient is obtained when they overlap perfectly, *i.e.*, when the ratio between analyte retention volume and gradient retention volume, $V_{R,A}/V_{R,G}$, is 1.00 (*cf.*, Fig. 1).

Anion effects in eluent without probe

In a previous study, it was found that the simultaneously injected anion had significant effects on the analyte retention volume even when it was well separated from the analyte on elution [5]. It was also observed that the peak compression effect was disturbed when the anion was eluted to close to the analyte. In this instance the cationic probe was present in the eluent. To determine the importance of the anion itself on the analyte retention volume and peak shape without any effect of the probe, an investigation was made with eluents lacking the probe.

Three cationic analytes were injected together with 5.0 m*M* decanesulphonate (Fig. 2a). The anion, detected by an RI detector, was eluted with a much longer

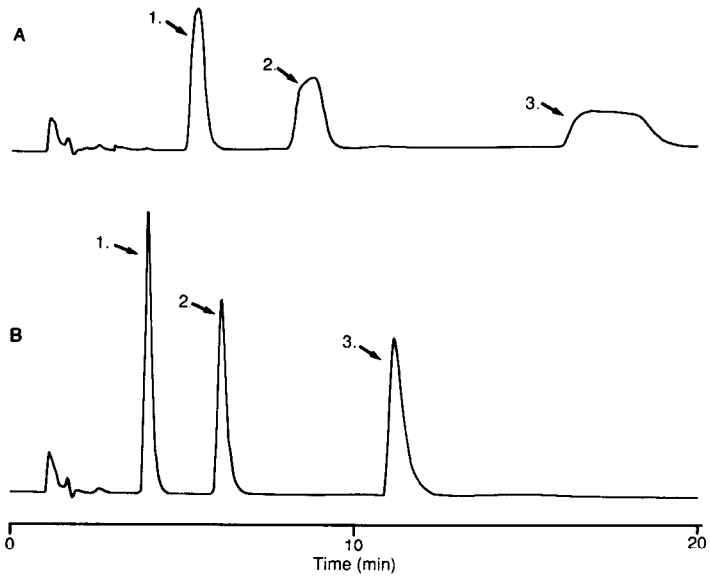

Fig. 2. Effects on analytes when injected simultaneously with a large amount of anion in a system lacking probe in the eluent. (A) Sample, $1.0 \cdot 10^{-5}$ *M* analytes and 5.0 m*M* decanesulphonate in eluent; eluent, phosphate buffer (pH 2.0)–acetonitrile (3:1). (B) As A, but without decanesulphonate in the sample. Analytes: 1 = FLA 731; 2 = FLA 913; 3 = FLA 797.

retention time than the analytes, in this instance about 25 min. The eluted analyte peaks were greatly deformed and the retention times and peak widths were significantly larger than in the isocratic experiment (*i.e.*, injection without organic anions in the sample); see Fig. 2b. The degree of deformation seems to be larger the closer the analyte peaks were eluted to the anion. However, even the weakly retarded analyte FLA 731, which had a five times lower retention time than the anion, was seriously affected. Its retention time increased from 4.0 to 5.4 min and its peak width from 0.40 to 0.72 min, compared with the isocratic experiment.

The effect on the amine analyte retention times of injected organic anions can be explained as ion-pairing effects in the starting zones and also in co-migrating zones in the column. In reversed-phase ion-pair chromatography, similar effects were utilized to determine if a solute is negatively or positively charged [22,23]. One or several injections of extremely high counter ion or co-ion concentrations were then made just before the sample injection. Solute–solute interactions discussed in the literature usually describe the situation in preparative chromatography, when the eluted components more or less overlap with each other [24,25].

Anion effects in eluent with probe

The analyte was injected together with the anion at different probe concentrations in the eluent. The analyte eluted just after the probe gradient induced by the anion, but before the anion (Table I). $\Delta V_{R,A}$ is the difference in the analyte retention volumes between the injections with and without anions. At low probe concentrations, the increases in analyte retention volume and peak width were significant. The effects decreased with increasing probe concentration and were negligible at the highest probe concentration. The increased probe concentration was accompanied by an improved anion separation from the analyte. At the highest probe concentration, a less hydrophobic anion was injected, as otherwise the retention volume of the anion would be unsuitably large [7].

TABLE I

INFLUENCE ON ANALYTE RETENTION AND PEAK WIDTH OF THE SIMULTANEOUSLY INJECTED ORGANIC ANION AT DIFFERENT PROBE ELUENT CONCENTRATIONS

The analyte was eluted between the system peak and the anion peak. Sample: $1.0 \cdot 10^{-5}$ *M* analyte with or without 5.0 m*M* anion in phosphate buffer (pH 2.0). Eluent: protriptyline (PT) in phosphate buffer (pH 2.0)–acetonitrile (3:1). $\Delta V_{R,A}$ = Analyte retention volume difference: injection with anion minus the isocratic experiment (without anion). $w_b/w_{b,iso}$ = Relative analyte peak width: injection with anion relative to the isocratic experiment.

PT (*M*)	Analyte and anion	$\Delta V_{R,A}$ (ml)	$\frac{w_b}{w_{b,iso}}$	$\frac{V_{R,anion}}{V_{R,analyte}}$
$7.6 \cdot 10^{-6}$	Imipramine and decanesulphonate	4.5	1.6	1.1
$3.8 \cdot 10^{-5}$	As above	3.5	1.4	1.3
$1.9 \cdot 10^{-4}$	As above	1.6	1.2	2.3
$9.5 \cdot 10^{-4}$	FLA 659 and octanesulphonate	0.0	1.0	2.1

The results show that the effects on the analyte caused by the anion in the starting and migrating zones were efficiently counteracted by the increased probe concentration. The reason is mainly increased competition due to the probe, especially the competition between the probe and the analyte for the organic anion. The interactions were also decreased by an increasing anion separation from the analyte.

Influence on analyte retention in the gradient

Peak compressions were obtained when the analyte was eluted together with the rear part of the negative system zone. The retention volume of the analyte was then greater than that in the isocratic experiment, owing to the low probe (co-ion) concentration in the negative zone. In addition, the initial ion-pair distribution with the organic anion will increase the retention volume.

The contributions from the two effects were determined qualitatively at different probe concentrations (Table II). $\Delta V_{R,A}$ is the analyte retention volume of the compressed analyte peak minus the value in the isocratic experiment. $\Delta V^*_{R,A}$ is the difference in isocratic analyte retention volumes in eluents lacking and containing the probe, respectively. The latter value is an estimate of the largest increase in analyte retention volume that could possibly be achieved owing to migration in a minimum probe concentration in the system zone. This value was only 0.4 ml at the lowest probe concentration, whereas the increase in the analyte retention volume under the peak compression conditions was 3.9 ml. This indicated a very large contribution from the hydrophobic anion, decanesulphonate. With increasing probe concentration, the influence of the low probe concentration in the system zone on the analyte retention volume increased. On the other hand, the contribution from the anion to the analyte retention volume decreased with increasing probe concentration (*cf.*, Table I). In the peak compression situation it may therefore be assumed that the contribution from the anion was very low at the probe concentrations corresponding to the non-linear part of the adsorption isotherm. The largest increase in retention volume (Table II) was

TABLE II

INFLUENCE OF DIFFERENT PROBE CONCENTRATIONS ON THE RETENTION OF COMPRESSED ANALYTES

Sample: $1.0 \cdot 10^{-5}$ *M* analyte with or without 5.0 m*M* anion in phosphate buffer (pH 2.0). Eluent: phosphate buffer (pH 2.0)–acetonitrile (3:1) with or without protriptyline (PT). $\Delta V_{R,A}$ = Compressed analyte retention volume minus isocratic retention volume (eluent containing probe). $\Delta V^*_{R,A}$ = Isocratic analyte retention volume in eluent lacking probe minus isocratic retention volume in eluent containing probe.

PT (*M*)	Analyte and anion	$\Delta V_{R,A}$ (ml)	$\Delta V^*_{R,A}$ (ml)	$\frac{V_{R,anion}}{V_{R,analyte}}$
$7.6 \cdot 10^{-6}$	Desipramine and decanesulphonate	3.9	0.4	1.2
$3.8 \cdot 10^{-5}$	Desipramine and nonylsulphate	4.6	1.6	1.5
$1.9 \cdot 10^{-4}$	FLA 659 and decanesulphonate	2.2	4.8	2.6
$9.5 \cdot 10^{-4}$	FLA 870 and octanesulphonate	2.3	5.9	2.5

obtained at a probe concentration of $3.8 \cdot 10^{-5}$ M (strong site covered to 12%). This is probably due to a combination of a considerable contribution from the anion injected together with the effect due to the low probe concentration in the system zone. At the two highest probe concentrations the $\Delta C/C_b$ values were 0.7, indicating that the analyte retention volume could be further increased by an additional lowering of the probe concentration in the gradient.

This is in line with previous results which indicated that the ion-pair distribution with the anion substantially influenced the retention volume of the gradient at low probe concentrations [7]. It is obvious that eqn. 1 is not qualitatively valid when the cationic analyte is eluted together with the gradient at low probe concentrations in the eluent. According to the significant initial ion-pair distribution with the organic anion, the equation may be modified to the expression below for a qualitative description. In this equation, the competing effect from the probe is assumed to be negligible.

$$V_{N,HA} \approx W_s K_0 (K_{HAX}[X^-]_m + K_{HAZ}[Z^-]_m) \quad (2)$$

where X^- is a buffer component in the eluent and Z^- is the simultaneously injected organic anion. It is important to note that the term corresponding to the organic anion is only significant in the injection zone and during migration of unresolved analyte and anion zones. Further, the concentrations of both X^- and Z^- will vary during the migration of the analyte zone together with the anion and system zones. The equation can therefore, only be used to indicate the different parameters that are of importance for the analyte retention. At increasing probe eluent concentrations, the retention gradually becomes governed by the unmodified eqn. 1.

Matching

In order to obtain the conditions such that the analyte and the gradient are eluted together, an appropriate choice of the eluent amine is necessary. Rough matching of the analyte and gradient retention volumes can then be made by a careful variation of the probe concentration [7]. If the analyte is eluted close to the gradient, fine matching can be performed by changing the concentration of the anion, injected together with the analyte [5]. An alternative for fine matching is to change the nature of the injected anion, as demonstrated below.

Fine matching. As illustrated above, both the analyte retention volume and the gradient retention volume are influenced by the ion-pair distribution with the organic anion at low probe concentrations in the eluent [7]. The analyte desipramine was injected together with anions of different hydrophobicities at a low probe concentration in the eluent, *i.e.*, $3.8 \cdot 10^{-5}$ *M*. When the analyte was injected with decanesulphonate, a small part of the analyte front was eluted before the gradient (see Fig. 3). Changing the anion to nonylsulphate resulted in both increased gradient and analyte retention volumes. However, the increase in analyte retention volume was larger, resulting in an improved matching and therefore a narrower analyte peak. The analyte peak width decreased from 0.55 to 0.45 ml. When the even more hydrophobic anion decylsulphate was used, the gradient retention volume increased more than the analyte retention volume and the peak compression effect was lost, resulting in an increased peak width, *i.e.*, 0.85 ml. In this instance, the lower peak compression effect was also due to a lower gradient steepness (see below).

At high probe concentrations, both analyte and gradient retention volumes were determined by the non-linear adsorption behaviour of the probe [7]. When the depth of the system peak or zone was constant despite increasing concentrations of the anion injected, the matching giving peak compression could be maintained when the anion concentration was increased up to fivefold (*cf.*, Fig. 10b and c). In addition, at higher probe concentrations it was possible to compress analytes with more varying isocratic retention volumes compared with the case with low probe concentrations.

The results indicate that fine matching is important at both low and high probe concentrations. However, at high probe concentrations the analyte peak is more easily captured in the gradient. This effect can be described in the light of eqn. 1 by the term in the denominator containing the probe concentration. When the analyte zone migrates within the low co-ion concentration in the system zone, it will have a lower migration speed than the system zone. When the analyte zone reaches the high co-ion concentration in the rear part of the system zone (the gradient), the rapidly increasing competition for adsorption sites results in a faster migration speed of the analyte zone. The analyte is caught in the gradient and a compressed peak results.

Dependence of gradient steepness on peak compression

The gradient steepness, $\Delta C/\Delta V$, an important parameter for peak compression, is dependent on both zone depth and gradient width (*cf.*, Fig. 1). Maximum steepness at a certain probe concentration is achieved when the probe concentration in the negative system zone approaches zero, *i.e.*, $\Delta C/C_b = 1.0$ [5]. The rear gradient steepnesses of the system zone or peak, obtained by the injection of 5.0 m*M* decanesulphonate at different probe concentrations, are shown in Table III. With increasing probe concentration, the depth of the system zone or peak increased and the gradient width decreased, resulting in a larger gradient steepness. The magnitude of the equilibrium disturbances decreased with increasing probe concentration [7], indicating possibilities of increasing the gradient steepness further. The narrower gradient width at higher probe concentrations is a result of the non-linear adsorption behaviour [26].

When decylsulphate was used instead of decanesulphonate at the lowest probe concentration in Table III, the gradient width was larger, which resulted in a lower gradient steepness (*cf.*, Fig. 3).

Peak compression effects obtained at different probe eluent concentrations are illustrated in Fig. 4. The compressed analyte peak width and the corresponding gradient width are plotted *versus* the probe concentration in the eluent. The analyte

TABLE III

DEPENDENCE OF GRADIENT STEEPNESS ON PROBE ELUENT CONCENTRATION

Sample: 5.0 m*M* decanesulphonate in phosphate buffer (pH 2.0). Eluent: Protriptyline (PT) in phosphate buffer (pH 2.0)–acetonitrile (3:1).

Bulk concentration of PT (*M*)	Depth, ΔC (*M*)	Gradient width, ΔV (ml)	Gradient steepness, $\Delta C/\Delta V$ (*M*/ml)
$3.8 \cdot 10^{-5}$	$3.8 \cdot 10^{-5}$	0.35	$1.1 \cdot 10^{-4}$
$1.9 \cdot 10^{-4}$	$1.3 \cdot 10^{-4}$	0.16	$8.1 \cdot 10^{-4}$
$9.5 \cdot 10^{-4}$	$3.5 \cdot 10^{-4}$	0.13	$2.7 \cdot 10^{-3}$

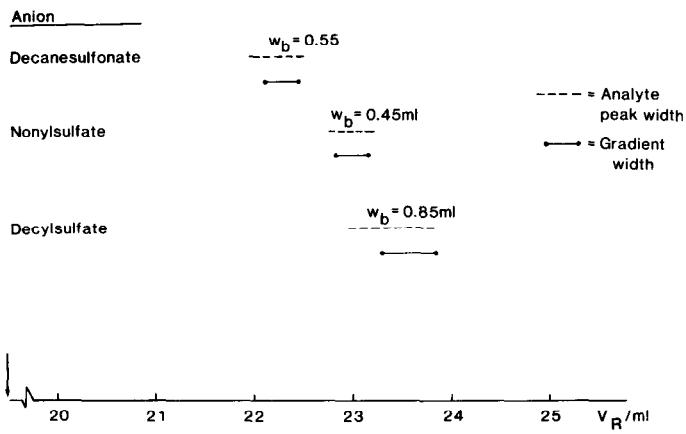

Fig. 3. Fine matching of analyte and gradient retention volumes. The character of the simultaneously injected anion was changed. Sample, $1.0 \cdot 10^{-5}$ *M* desipramine and 5.0 m*M* anion in phosphate buffer (pH 2.0); eluent, $3.8 \cdot 10^{-5}$ *M* protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1).

peaks were more strongly compressed at higher probe concentrations (larger gradient steepness). At the two highest probe concentrations the peak heights increased several times and the peak widths decreased 4–5-fold (compared with the isocratic experiments).

In each peak compression situation in Fig. 4 the degree of matching was optimum according to the definition $V_{R,A}/V_{R,G} = 1.00$. However, perfect overlapping

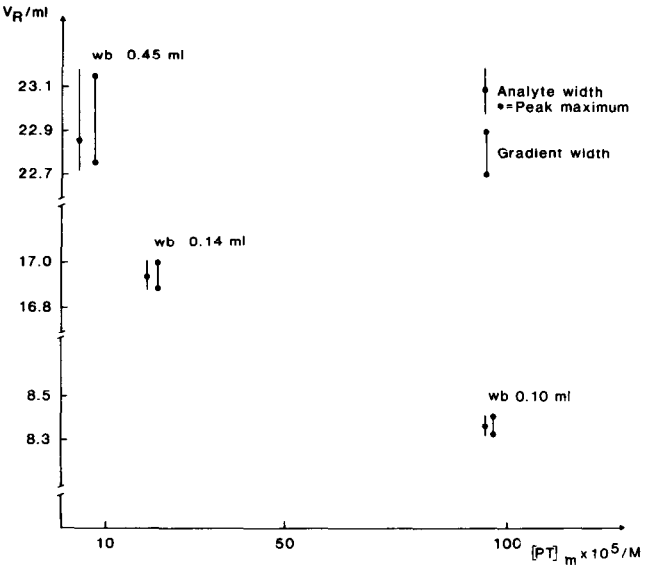

Fig. 4. Dependence of peak compression on probe eluent concentration. Sample, $1.0 \cdot 10^{-5}$ *M* analyte and 5.0 m*M* anion in phosphate buffer (pH 2.0); eluent, protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1). The analyte–anion pair used at the respective PT concentration are given in Table II.

of compressed analyte peak widths with the respective gradient widths were obtained only at the two highest probe concentrations. This indicates that the gradient width is a key parameter in order to obtain efficient peak compression effects. The gradient width is expected to decrease with increasing column efficiency [27], which is probably another important parameter for optimum peak compressions, which, however, has not yet been studied systematically.

Analyte peak deformations in the gradient

When the analyte peak was eluted together with the front part of the negative system peak, an analyte peak deformation developed [5]. The analyte peak then eluted in a decreasing probe gradient, resulting in broadening of the peak (*cf.*, Fig. 11). However, if the analyte is eluted closer to the rear gradient, this will result in an improved peak shape. When the analyte is eluted very close to the rear gradient, peak deformation seems to be avoided. An example is shown in Fig. 5, where the shape and width of the eluted analyte peak are comparable to those in the isocratic experiment.

Even with analytes in the position normally giving peak compression, deformations may appear, *e.g.*, when the co-injected anion was eluted too close after the gradient and/or when high analyte concentrations were injected [5]. The conditions responsible for peak deformation in this position were investigated carefully, in order to find guidelines for avoiding their appearance in the design of separations where peak compression is desired.

Deformation at low analyte concentration. In Table I it was demonstrated that the tendency for the anion to affect the analyte peak shape, *i.e.*, increase in peak width, decreased with increasing probe concentration. When the analyte was eluted together with the gradient, the tendency of the anion to increase the analyte peak width was further counteracted by the compression effect. At the lowest probe concentration at which peak compression was demonstrated in Fig. 4, *i.e.*, $3.8 \cdot 10^{-5}$ *M*, the retention volume of the compressed peak was largely affected by the ion-pair distribution with the anion (*cf.*, Table II). Despite this, the peak shape seemed to be unaffected and an adequate peak compression effect was obtained. The ratio between the anion and the

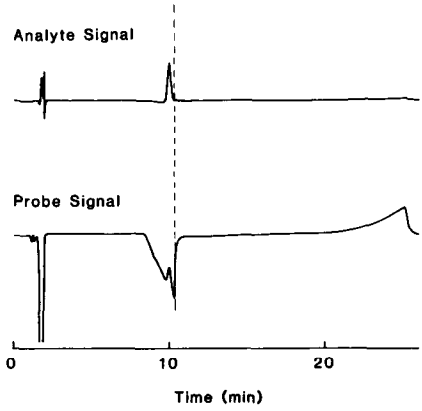

Fig. 5. Effects on the analyte peak shape when it was eluted together with the front part but very close to the rear part gradient of the system peak. Sample, $1.0 \cdot 10^{-5}$ *M* FLA 870 and 3.0 m*M* octanesulphonate in phosphate buffer (pH 2.0); eluent, $9.1 \cdot 10^{-4}$ *M* protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1).

analyte retention volumes was 1.5. When the probe concentration was further decreased, giving the above ratio of 1.2, the analyte peak showed significant tailing caused by the closely eluting anion (see Fig. 6). At this low probe concentration, where the peak compression effect was also low, the analyte peak deformations due to the more retained anion (cf., Fig. 2a) will be more pronounced.

The anion front is often broad and deformed owing to its initial migration together with the gradient [7]. At increasing probe concentration, the tendency of the gradient to deform the later eluted anion peak increased and ultimately resulted in a deformed gradient. When the analyte peak was eluted in such a deformed probe gradient, a tailed and deformed analyte peak resulted instead of a compressed one (Fig. 7). In this instance, the analyte signal was registered at 343 instead of 350 nm (see Experimental). Therefore, the disturbance of the analyte signal due to the high probe absorbance was pronounced and contributed to the deformed shape of the analyte peak.

In conclusion, it is essential to have an adequate selectivity between the large anion peak and the gradient, irrespective of whether the probe eluent concentration is low or high.

Deformation at high analyte concentration. When the analyte concentration was increased tenfold, *i.e.*, from $1.0 \cdot 10^{-5}$ to $1.0 \cdot 10^{-4}$ *M*, the peak compression effect disappeared and instead analyte peak deformation and splitting developed [5]. This deformation occurred when the analyte was still eluted in the probe gradient, which was expected to result in peak compression. The probe signal was also deformed at the corresponding retention volume (the gradient). Fig. 8a shows that this kind of deformation arose when $1.0 \cdot 10^{-4}$ *M* of the analyte was injected and the probe concentration was $3.8 \cdot 10^{-5}$ *M*. When the probe concentration was increased fivefold, the analyte peak was compressed and the degree of deformation of both the analyte peak and the gradient was less significant (Fig. 8b). When the analyte concentration

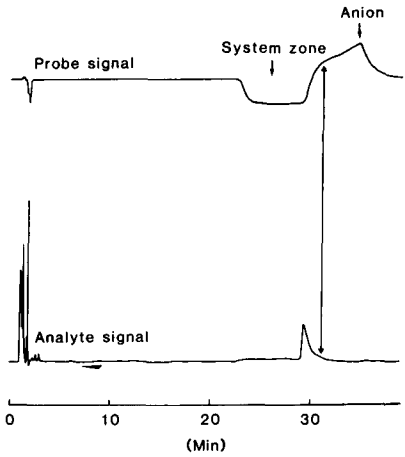

Fig. 6. Tailing effects on the compressed analyte peak by the closely eluted anion. Sample, $1.0 \cdot 10^{-5}$ *M* desipramine and 5.0 m*M* decanesulphonate in phosphate buffer (pH 2.0); eluent, $7.6 \cdot 10^{-6}$ *M* protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1).

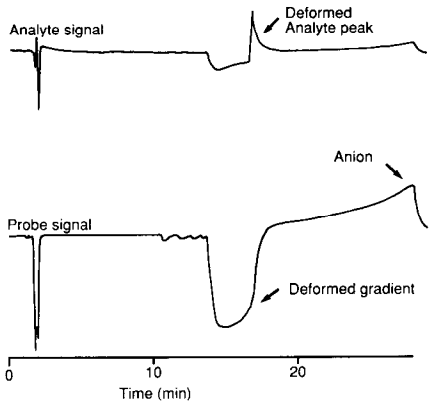

Fig. 7. Effects on the analyte peak when eluted in a deformed gradient. Sample, $1.0 \cdot 10^{-5}$ *M* FLA 965 and 5.0 m*M* nonanesulphonate in phosphate buffer (pH 2.0); eluent, $1.9 \cdot 10^{-4}$ *M* protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1). An unsatisfactory compensation of the analyte signal can be seen.

was increased further, using the same high probe concentration, a deformed peak again developed.

Similar effects arose also in eluents without the probe. The analytes injected in the run shown in Fig. 2a were injected at a tenfold higher concentration, *i.e.*, $1.0 \cdot 10^{-4}$ *M*, together with 5.0 m*M* decanesulphonate into a system where the eluent consisted of only a buffer–acetonitrile mixture (Fig. 9). The anion retention time was 25.5 min. The analyte peaks were extremely broad and deformed, despite the relative large separation from the anion. The greater deformation compared with the experiment where lower

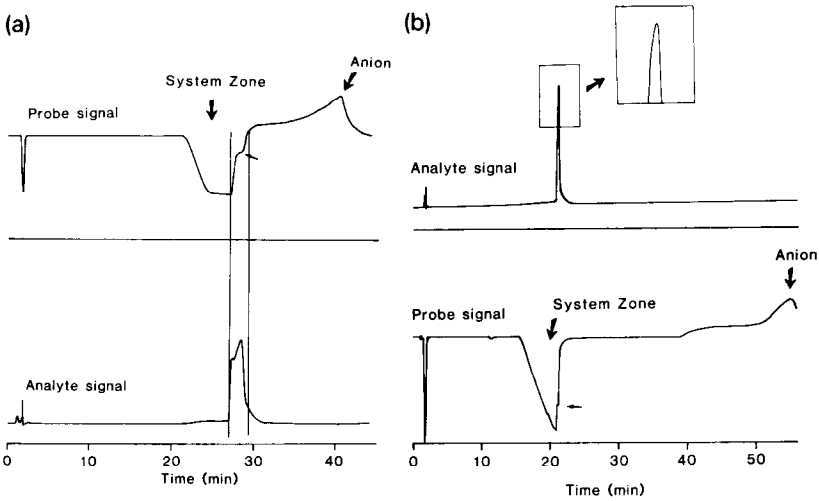

Fig. 8. Deformations when high analyte concentrations were eluted together with the gradient. Sample, $1.0 \cdot 10^{-4}$ *M* analyte and 5.0 m*M* decanesulphonate in phosphate buffer (pH 2.0); eluent, protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1). (a) $C_{PT} = 3.8 \cdot 10^{-5}$ *M*, analyte desipramine; (b) $C_{PT} = 1.9 \cdot 10^{-4}$ *M*, analyte FLA 659.

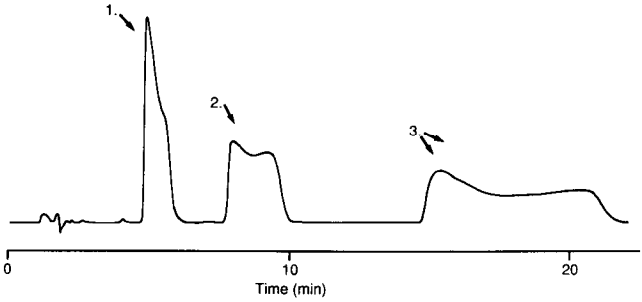

Fig. 9. Anion effects at high analyte concentrations with eluent lacking probe. Sample and eluent as in Fig. 2a except that the concentrations of the analytes were $1.0 \cdot 10^{-4}$ *M*.

analyte concentrations were injected (*cf.*, Fig. 2a) was due to an increased interaction between the starting and unresolved analyte and anion zones. On the other hand, even when peak compression was induced by means other than the injection of an organic anion, deformation developed at higher analyte concentrations injected [28].

The principal factors which cause peak deformation when high analyte concentrations are eluted in the gradient are of at least two types: more extensive analyte–anion interactions and a lower ability of the probe to displace the analyte. The contributions from both of these factors are counteracted by increasing the eluent probe concentration. It should be noted that the contribution from the anion on deformation of both low and high analyte concentrations was not properly taken into consideration in a previous paper [5].

Aspects of selectivity

Several analytes, the benzamides FLA 870, FLA 965 and FLA 659, desipramine, imipramine and nortriptyline (in order of increasing retention volumes), were injected together with 5.0 m*M* nonylsulphate (Fig. 10a). The column was equilibrated with a low probe concentration of $3.8 \cdot 10^{-5}$ *M*. FLA 870 and FLA 965 were eluted in the front and the plateau of the system zone, respectively, and were both deformed. The simultaneously injected anion may also be partly responsible for this deformation (*cf.*, Fig. 2a). FLA 659 and desipramine (isocratic separation factor = 1.02) were both eluted in the gradient and appeared as a single compressed peak. The analytes which were eluted after the gradient were deformed owing to overlap with the anion front. In a second experiment, the substituted benzamides were injected together with octanesulphonate when the eluent contained a high probe concentration of $9.5 \cdot 10^{-4}$ *M* (Fig. 10b). In this instance the analytes FLA 870 and FLA 965, with an isocratic separation factor of 1.29, were eluted with the same retention volumes and appeared as a single compressed peak. The retention volume and peak shape of FLA 659, which was eluted after the gradient, were identical with those in the isocratic experiment. Separation between FLA 870 and FLA 965 could be achieved when a fivefold higher anion concentration was used, *i.e.*, 25 m*M* (Fig. 10c). At this high anion concentration, the gradient retention volume was decreased [7], accompanied by FLA 870, which was still compressed. The retention volumes of FLA 965 and FLA 659 were increased owing to an increased ion-pair distribution with the anion. In this run and also in the run in Fig. 10b, the retention time of the later eluted anion peak was about 25 min.

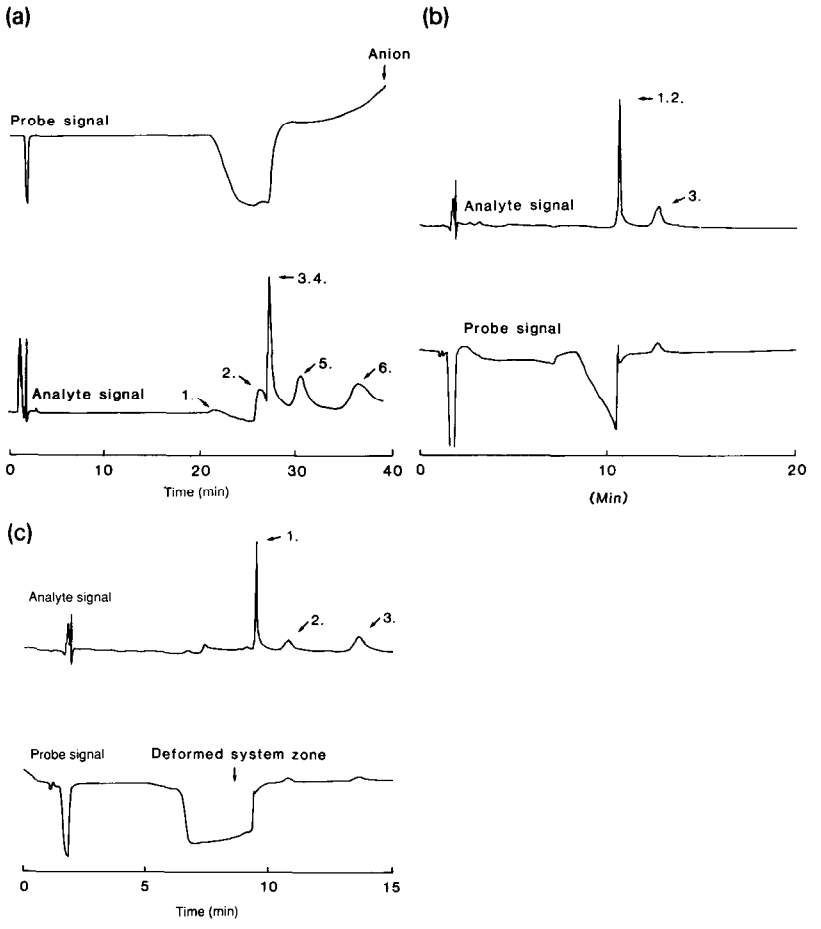

Fig. 10. Selectivity of peak compression, induced at different probe eluent concentrations. Sample, $1.0 \cdot 10^{-5}$ *M* analytes and anion in phosphate buffer (pH 2.0); eluent, protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1). (a) Analytes: 1 = FLA 870; 2 = FLA 965; 3 = FLA 659; 4 = desipramine; 5 = imipramine; 6 = nortriptyline. Anion: 5.0 m*M* nonylsulphate. $C_{PT} = 3.8 \cdot 10^{-5}$ *M*. (b) Analytes: 1–3 as in (a). Anion: 5.0 m*M* octanesulphonate. $C_{PT} = 9.5 \cdot 10^{-4}$ *M*. (c) Conditions as in (b), except 25.0 m*M* octanesulphonate.

This series of results indicate that high probe concentrations in the eluent give low selectivity between the amine analytes. However, there are possibilities of achieving the desired selectivity between the analytes by adjustments of the anion concentration. It is further obvious that by this peak compression technique only one component at a time can be compressed. An important advantage of the use of high probe concentrations is the improved separation between the gradient and the anion, which is necessary to avoid deformations of analytes being eluted after the gradient.

Other ways to induce system peaks

In the experiments described above, the analytes and anions were dissolved in

phosphate buffer. The injection of pure phosphate buffer containing no organic ions results in positive system peaks [17]. The injection of eluent without a probe, *i.e.*, phosphate buffer–acetonitrile (3:1), into the system equilibrated with the probe resulted in a negative system peak, because in the injection zone some of the PT molecules adsorbed to the solid phase will diffuse into the injected solvent. This leaves a deficiency of the probe (PT) in the injection zone to be eluted as a negative system peak.

By use of this technique, the analytes FLA 870, FLA 965 and FLA 659 were injected (Fig. 11). The probe concentration was $9.5 \cdot 10^{-4}$ *M*. None of the analytes were clearly eluted within the rear gradient of the system peak, but the peak width of FLA 965 decreased from the isocratic value of 0.52 ml to 0.34 ml. This was due to its elution very close after the gradient. The poorly retarded FLA 870 was partly eluted with the front part of the system peak and was therefore strongly deformed, whereas the analyte FLA 659, which was eluted well separated after the rear gradient, was unaffected.

This demonstrates the importance of careful control of the injection conditions in order to avoid disturbances in ion-pair chromatographic systems. The results also indicate that it is possible to achieve peak compressions without the simultaneous injection of an organic anion [28].

Reproducibility of peak compression

Limited studies on the reproducibility of the technique were made by a comparison of single injections made several weeks apart (Table IV). The retention volume of the compressed analyte peak and also the matching of analyte and gradient retention volumes remained almost constant after more than 7 weeks of running the system. With desipramine, the analyte retention volume increased very slightly from 22.86 to 23.05 ml, while the matching was still optimum. Tendencies towards higher retentions

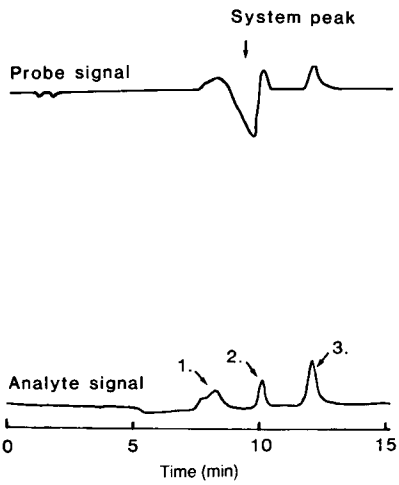

Fig. 11. System peak effects on analytes induced by injecting small amounts of analytes dissolved in eluent lacking probe. Sample, $1.0 \cdot 10^{-5}$ *M* analytes 1–3 as in Fig. 10 in eluent without probe; eluent, $9.5 \cdot 10^{-4}$ *M* protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1).

TABLE IV

REPRODUCIBILITY OF PEAK COMPRESSION: COMPARISON OF SINGLE INJECTIONS MADE SEVERAL WEEKS APART

Sample: $1.0 \cdot 10^{-5}$ *M* analyte with 5.0 m*M* anion in phosphate buffer (pH 2.0). Eluent: protriptyline in phosphate buffer (pH 2.0)–acetonitrile (3:1). Systems: (A) analyte FLA 870, anion octanesulphonate, $C_{PT} = 9.5 \cdot 10^{-4}$ *M*; (B) analyte desipramine, anion nonylsulphate, $C_{PT} = 3.8 \cdot 10^{-5}$ *M*.

System	$V_{R,A}$ (ml)	$V_{R,A}/V_{R,G}$	w_b (ml)
A	8.37 } same day	1.00	0.09
	8.38 }	1.00	0.10
	8.41 (7 weeks later)	1.00	0.13
B	22.86	1.00	0.45
	23.05 (3 weeks later)	1.00	0.20[a]

[a] A small amount of new solid phase was added on the top of the column.

in similar systems have been observed before [18]. The slight changes in the retention volume of the compressed analyte peaks followed the changes in gradient retention volume, maintaining the good matching of the peaks. The differences in the compressed peak widths obtained was probably a result of similar changes in the gradient widths (gradient steepness). Further studies on reproducibilities in order to adapt the technique for quantitative determinations are in progress.

CONCLUSIONS

Peak compression is optimum at high probe concentrations (non-linear distribution behaviour), giving large gradient steepnesses at low analyte concentrations and when an adequate separation between the system peak and the anion peak is obtained. Peak deformation was obtained in the position normally giving peak compression when the anion peak eluted close after the system peak and interfered with the analyte. Such interferences were efficiently suppressed by increased probe concentrations and improved anion separations.

The analyte desired for peak compression was more easily captured in the gradient at high probe concentrations. A single compressed peak may then appear when two analytes are eluted simultaneously together with the gradient, but the components can be separated by suitable adjustments of the injected anion concentration.

ACKNOWLEDGEMENTS

The authors thank the Swedish Natural Science Research Council (NFR) and Pharmacia LEO Therapeutics for financial support of this project. We also thank the Swedish Academy of Pharmaceutical Sciences (T.F.) and the I.F. Foundation of Pharmaceutical Research (T.F. and A.S.) for travel grants and Astra Research Centre for supplying the substituted benzamides.

REFERENCES

1 P. Jandera and J. Churacek, *Gradient Elution in Column Liquid Chromatography (Journal of Chromatography Library*, Vol. 31), Elsevier, Amsterdam, 1985.
2 A. Sokolowski, *Chromatographia*, 22 (1986) 177.
3 A. Sokolowski, *J. Chromatogr.*, 384 (1987) 1.
4 A. Sokolowski, *J. Chromatogr.*, 384 (1987) 13.
5 T. Fornstedt, D. Westerlund and A. Sokolowski, *J. Liq. Chromatogr.*, 11 (1988) 2645.
6 L. B. Nilsson and D. Westerlund, *Anal. Chem.*, 57 (1985) 1835.
7 T. Fornstedt, D. Westerlund and A. Sokolowski, *J. Chromatogr.*, 506 (1990) 61.
8 G. Schill and E. Arvidsson, *J. Chromatogr.*, 492 (1989) 299.
9 T. Arvidsson, *J. Chromatogr.*, 407 (1987) 49.
10 S. O. Jansson and M. L. Johansson, *J. Chromatogr.*, 395 (1987) 495.
11 W. S. Hancock, C. A. Bishop, J. E. Battersby and D. R. K. Harding, *J. Chromatogr.*, 168 (1979) 377.
12 H. U. Ehmcke, H. Kelker, K. H. König and H. Ollner, *Fresenius' Z. Anal. Chem.*, 294 (1979) 251.
13 G. K. C. Low, A. M. Duffield and P. R. Haddad, *Chromatographia*, 15 (1982) 289.
14 J. Kirschbaum, S. Perlman and R. B. Poet, *J. Chromatogr. Sci.*, 20 (1982) 336.
15 T.-L. Ng and S. Ng, *J. Chromatogr.*, 329 (1985) 13.
16 G. K. C. Low and P. R. Haddad, *J. Chromatogr.*, 336 (1984) 15.
17 A. Sokolowski, T. Fornstedt and D. Westerlund, *J. Liq. Chromatogr.*, 10 (1987) 1629.
18 A. Sokolowski, *Chromatographia*, 22 (1986) 168.
19 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299.
20 A. Tilly-Melin, M. Ljungcrantz and G. Schill, *J. Chromatogr.*, 185 (1979) 225.
21 L. R. Snyder, *Anal. Chem.*, 39 (1967) 698.
22 V. V. Berry and R. E. Shansky, *J. Chromatogr.*, 284 (1984) 303.
23 G. K. C. Low, *J. Chromatogr.*, 478 (1989) 21.
24 F. Eisenbeiss, S. Ehlerding, A. Wehrli and J. F. K. Huber, *Chromatographia*, 20 (1985) 657.
25 A. Katti and G. Guiochon, *Anal. Chem.*, 61 (1989) 982.
26 J. R. Conder and C. L. Young, *Physicochemical Measurement by Gas Chromatography*, Wiley, New York, 1979.
27 C. N. Reilley, G. P. Hildebrand and J. W. Ashley, *Anal. Chem.*, 34 (1962) 1198.
28 T. Fornstedt, A. Sokolowski and D. Westerlund, presented at the *13th International Symposium on Column Liquid Chromatography, Stockholm, June 25–30, 1989.*