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Gradient elution with shorter equilibration times in reversed-phase ion-pair chromatography

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ABSTRACT

To reduce the re-equilibration time needed between gradient runs in reversed-phase ion-pair chromatography (RP-IPC), various chromatographic conditions were studied. Five pairing ions of different size (and sorption kinetics), *viz.*, trifluoroacetate, heptafluorobutyrate, octylsulphonate, dodecyl sulphate and tetrabutylammonium, each at two different concentrations, and three types of gradient (acetronitrile, salt and mixed gradients) were examined. Some common nucleotides, such as adenosine-5′-monophosphate, adenosine-5′-diphosphate and adenosine-5′-triphosphate, and some primary amines, such as dopamine, α-methyldopamine and serotonin, were used as analytes. The "efficiency" of the various gradient systems was tested in terms of the "speed" of re-equilibration and the average decrease in retention time achieved. Without solving the general problem of slow equilibration in ion-pair gradient elution, chromatographic conditions are recommended under which 1–3 column volumes of solvent A can be sufficient for re-equilibration between gradient runs, *i.e.*, for obtaining reproducible retention times in gradient RP-IPC.

INTRODUCTION

Isocratic reversed-phase ion-pair chromatography (RP-IPC) has become a popular method for the separation of ionic, ionizable and neutral compounds. Gradient RP-IPC, however, has not gained equal acceptance and has been associated with long run times owing to the need for restoring the initial ionic equilibria on the surface of the reversed-phase packing material. It is generally suggested that of reversed-phase, normal-phase (polar bonded phases), ion-exchange and RP-IPC systems, the last is least suited for gradient elution, requiring more than 15–20 column volumes of solvent A for column regeneration [1], which is not practical for most routine applications.

To understand our approach aimed at reducing re-equilibration volumes in gradient RP-IPC, brief comments on the basis of RP-IPC separations may be appropriate. As discussed recently by Liu and Cantwell [2], retention in RP-IPC is governed both by dynamic ion-exchange (in the diffuse part of the electrical double layer formed on the hydrophobic surface of the stationary phase owing to the selective adsorption of the pairing ion used) and adsorption on the electrically charged surface. In view of the retention of ionic solutes, the surface concentration of the pairing ion is a parameter of major importance as it largely determines both the "ion-exchange capacity" of the system and the electric potential on the surface. Reproducibility of retentions in RP-IPC is, therefore, closely related to the reproducibility of the surface concentration of the pairing ion (on which the reproducibility of the electrical double layer also depends).

In this paper, we are concerned with the "speed" of equilibration in gradient RP-IPC, which is synonymous with the "speed" of restoring the initial surface concentration of the pairing ion after a gradient run. To increase that "speed", the following means can be considered: (i) choosing pairing ions with good kinetic properties; (ii) choosing pairing ion concentrations at which the adsorption isotherm in the RP-IPC system concerned starts to flatten out or which fall on the plateau region of the isotherm; and (iii) keeping the change in pairing ion

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surface concentrations to the necessary minimum during a gradient run.

As kinetic properties can vary considerably, depending on the size, and even shape, of the pairing ion used [3], we tested five pairing ions of different size, *viz.*, trifluoroacetate (TFA), heptafluorobutyrate (HFBA), octylsulphonate (SOS), dodecyl sulphate (SDS) and tetrabutylammonium (TBA), at two different eluent concentrations. At pairing ion concentrations that fall on the flattening portion or the plateau region of the adsorption isotherm there is relatively more pairing ion in the mobile phase (than in the stationary phase) to drive the system towards attaining equilibrium in a shorter equilibration time (by a smaller volume of solvent A).

Concerning consideration (iii), it should be mentioned that two main RP-IPC gradient principles can be used [4,5]: partial desorption of the pairing ion by a gradual increase in the percentage of the organic modifier (*e.g.*, acetonitrile) in the eluent; and increasing the counter ion concentration or ionic strength in the eluent by a gradual increase in the (buffer) salt concentration in the mobile phase. The first principle, which has gained a much wider use, is known to affect pairing ion surface concentrations considerably (organic modifiers can change the shape [6] and even modify the type [7] of the adsorption isotherm of a pairing ion). The second principle (salt gradients) reduces the retention of ionic solutes through ion-exchange effects in the diffuse part of the electrical double layer and, as a result, causes relatively smaller changes in pairing ion surface concentrations.

The aim of this work was to test the potential means by which the re-equilibration time between RP-IPC gradient runs can be substantially reduced. Three types of RP-IPC gradient (acetonitrile, salt and acetonitrile–salt mixed type) with each pairing ion were examined and compared in terms of "efficiency", *i.e.*, the "speed" of re-equilibration and the average decrease in retention time achieved. Salt gradients and mixed gradients (at a suitable eluent concentration of HFBA, SOS or TBA as pairing ion) proved to be "efficient", resulting in reproducible gradient retention times after passing only 1–3 column volumes of solvent A through the column between gradient runs.

EXPERIMENTAL

Apparatus and materials

The apparatus used for the RP-IPC experiments was an LKB (Bromma, Sweden) gradient system consisting of two Model 2150 pumps, a Model 2152 controller, a Model 2151 variable-wavelength monitor (at 254 and 275 nm for nucleotides and amines, respectively), a Model 2210 two-channel recorder and a Rheodyne (Cotati, CA, USA) Model 7125 loop injector. The same type of packing material (Nucleosil 100-5 C_{18}, 5 μm, Macherey–Nagel, Düren, Germany) was used throughout the experiments. The BET surface area and the carbon content of the packing material were 310 m^2/g and 14% (w/w), respectively, as specified by the manufacturer. The chromatographic support was packed in stainless-steel columns by Bioseparation Technologies (Budapest, Hungary). The column dimensions were 150 × 4 mm I.D., with a 20 × 4 mm I.D. precolumn. The void volume of the column (V_0) was 1.63 ml (determined by the method of Deelder *et al.* [8]).

Sequanal-grade trifluoroacetic acid and heptafluorobutyric acid were obtained from Pierce (Rockford, IL, USA), sodium octylsulphonate and sodium dodecyl sulphate from Supelco (Bellefonte, PA, USA) and Aldrich (Steinheim, Germany), respectively, tetrabutylammonium bromide and dopamine hydrochloride (DA) from Sigma (St. Louis, MO, USA), adenosine-5′-monophosphoric acid (AMP), adenosine-5′-diphosphate (ADP) and adenosine-5′-triphosphate (ATP) from Calbiochem (La Jolla, CA, USA). Serotonincreatinine sulphate (5-HT) from Reanal (Budapest, Hungary) and α-methyldopamine hydrobromide (MDA) from Merck, Sharp and Dohme (West Point, PA, USA).

For the gas–liquid chromatographic (GLC) determination of TFA and HFBA, a Hewlett-Packard Model 5830 A gas chromatograph equipped with a flame ionization detector (Hewlett-Packard, Palo Alto, CA, USA) was used. The glass chromatographic column (6 ft. × 2 mm I.D.) was packed with Carbopack B (Supelco) coated with 0.3% (w/w) phosphoric acid and 3% (w/w) Carbowax 20M. The carrier gas was nitrogen at a flow-rate of 60 ml/min. The temperatures of the injection port, column and detector were 240, 200 and 240°C, respectively.

For the spectrophotometric determination of

SOS, SDS and TBA, methylene blue and methyl orange (Aldrich) were used. An LKB Ultrospec-II 4050 spectrophotometer was used to determine absorbances.

Acetonitrile, ethanol and chloroform (LiChrosolv) were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical-reagent grade.

Procedures

The compositions of the gradient systems studied are given in Table I. Solvent A for a given pairing ion (and concentration) was always the same, whereas solvent B was varied, as shown in Table I. [The letters A, S and M represent acetonitrile, salt and mixed gradients, respectively. The notations 1A, 2S, 3M, etc., refer to the respective curves in Figs. 2–11

TABLE I

COMPOSITION OF THE GRADIENT SYSTEMS STUDIED

Notation	Solvent A (with pairing ions)			Solvent B (difference from solvent A): type of gradient		
	pH	Components	Concentration	ACN[a]	Salt	Mixed
1A	2.50	TFA (Na^+)	8 m*M*	–	–	–
1S		Na^+ (phosphate)	15 m*M*	–	100	60
1M		ACN	6% (v/v)	15	–	10
2A	2.50	TFA (Na^+)	200 m*M*	–	–	–
2S		Na^+ (phosphate)	15 m*M*	–	100	60
2M		ACN	6% (v/v)	20	–	14
3A	2.50	HFBA (Na^+)	5 m*M*	–	–	–
3S		Na^+ (phosphate)	15 m*M*	–	100	60
3M		ACN	6% (v/v)	20	–	14
4A	2.50	HFBA (Na^+)	50 m*M*	–	–	–
4S		Na^+ (phosphate)	15 m*M*	–	100	60
4M		ACN	10% (v/v)	30	–	20
5A	2.50	SOS (Na^+)	1 m*M*	–	–	–
5S		Na^+ (phosphate)	15 m*M*	–	100	60
5M		ACN	10% (v/v)	20	–	15
6A	2.50	SOS (Na^+)	10 m*M*	–	–	–
6S		Na^+ (phosphate)	15 m*M*	–	100	60
6M		ACN	15% (v/v)	25	–	20
7A	2.50	SDS (Na^+)	0.5 m*M*	–	–	–
7S		Na^+ (phosphate)	15 m*M*	–	100	–
		ACN	20% (v/v)	35	–	–
8A	2.50	SDS (Na^+)	3 m*M*	–	–	–
8S		Na^+ (phosphate)	15 m*M*	–	100	–
		ACN	25% (v/v)	40	–	–
9A	5.80	TBA (Br^-)	5 m*M*	–	–	–
9S		$H_2PO_4^-$ (Na)	15 m*M*	–	100	60
9M		ACN	12% (v/v)	30	–	18
10A	5.80	TBA (Br^-)	60 m*M*	–	–	–
10S		$H_2PO_4^-$ (Na)	15 m*M*	–	150	100
10M		ACN	12% (v/v)	40	–	18

[a] ACN = Acetonitrile.

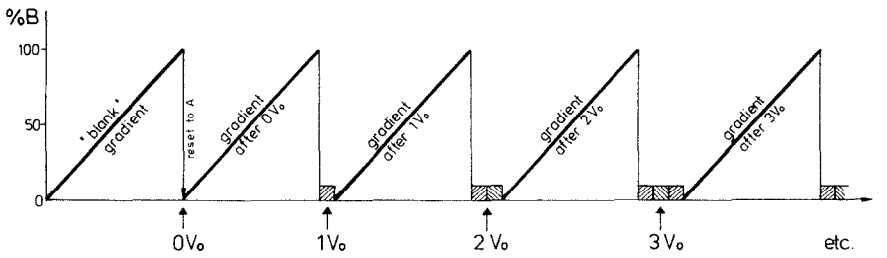

Fig. 1. Scheme for the preparation of graphs of k'_G *vs.* column volumes between runs. Hatched boxes represent one column volume (V_0) of solvent A between gradient runs.

and for Table III; they are of less relevance for Table I. By mixed gradients are meant gradients in which eluent strength is raised by acetonitrile and salt together, but the concentration of each (in solvent B of an M gradient) is lower than in solvent B of the respective A (acetonitrile only) or S (salt only) gradients.] All gradient experiments were carried out using the same linear gradient (from 100% A to 100% B in 15 min). The flow-rate and the separation temperature were 1 ml/min and 23 ± 1°C, respectively.

The graphs of k'_G *vs.* column volumes between runs were prepared as follows (for the definition of k'_G, an arbitrary term, see the text below). Following a 60-min equilibration with solvent A, the retention times of the test compounds were determined and the k'_0 values (the k' values in solvent A, determined in the usual way) for each compound were calculated. Then a "blank" gradient was run. After reaching 100% B and immediate resetting to solvent A, samples were injected (and a series of gradients started) at 0.00, 1.63, 3.26, 4.89, 6.52, 8.15, 16.30, 24.45 and 40.75 min after the resetting to solvent A. The time points indicated correspond to the time required to pass 0, 1, 2, 3, 4, 5, 10, 15 and 25 column volumes (V_0), respectively, of solvent A through the column before starting a certain gradient (the working scheme for the series of gradients is shown in Fig. 1). The retention times in each gradient run were measured and the k'_G values [the k' values obtained (by the usual equation) in a gradient performed after a specified number of V_0 of solvent A had been passed through the column] for each analyte calculated. The k'_G values thus obtained were plotted as a function of the V_0 used for re-equilibration before the respective gradient run. The steady-state k'_G values ($k'_{G,\text{"equilibrium"}}$) were read from the horizontal portions of the graphs.

The average retention decrease factors (ARDF values) for each gradient system were obtained as the average of the $k'_0/k'_{G,\text{"equilibrium"}}$ ratios of the compounds tested in the respective gradient system (*e.g.*, as shown in Table III, the ARDF value for gradient system 4A was 1.64, which was obtained as the average of the $k'_0/k'_{G,\text{"equilibrium"}}$ ratio for DA, MDA and 5-HT used as analytes in that system).

The gradient range ($\Delta\varphi$) for acetonitrile gradients is meant as defined in ref. 9 (*i.e.*, the change in the volume fraction of the organic eluent component between the start and end of a gradient run). It varied by a factor of *ca.* 3 (0.09–0.28) in order to elute the test compounds within the same gradient time (15 min). For salt gradients the gradient range means the change in total counter ion concentrations in the eluent during a gradient run. It was the same (85 mM) in all but one cases.

The surface concentrations of the pairing ions adsorbed by the stationary phase from the respective solvent A after a 60-min equilibration were determined as follows. For the determination of TFA and HFBA eluted after equilibration, the method of Di Corcia and Samperi [10] described for short-chain free acids was adopted, using HFBA as internal standard for TFA measurements, and *vica versa*. After passing one column volume of distilled water through the column (at a flow-rate of 0.25 ml/min) to remove much of the buffer, desorption of the pairing ion was carried out using acetonitrile as eluent (at a flow-rate of 0.5 ml/min). One 10-ml fraction and ten 2-ml fractions were collected. Aliquots of the collected fractions (containing the internal standard) were acidified with concentrated hydrochloric acid in a volume ratio of 2:1. Volumes of 2 μl were injected into the GLC system. To calculate surface concentrations, the amounts of

TFA (or HFBA) in the various fractions were added.

For the determination of SOS and SDS eluted after equilibration, the methylene blue method of Sharma *et al.* [11] was used (the volumes in the procedure were reduced by 4:1). After rinsing the column with one column volume of distilled water (0.25 ml/min), desorption of the pairing ion was carried out using 100 ml of absolute ethanol as eluent (0.5 ml/min). Three different aliquots of the eluate (in the volume range 0.02–0.10 ml), with parallels, were pipetted into Pyrex glass centrifuge tubes. After evaporating the solvent under a stream of nitrogen, the pairing ion was redissolved in 1 ml of distilled water and determined as described in ref. 11.

For the determination of TBA eluted (as described above for SOS and SDS) after equilibration, the methyl orange method of Simón *et al.* [12] was used with minor modifications (the volumes in the procedure were reduced by 2:1 and the Carmody buffer was replaced with 20 m*M* acetate buffer, pH 4). Three different aliquots of the eluate (in the volume range 0.02–0.20 ml), with paralles, were pipetted into Pyrex glass centrifuge tubes and used in the procedure [12] without evaporating the solvent (the volme of the ethanol to be added in the procedure was accordingly reduced by the volume of the aliquot added).

Precision values [relative standard deviation (R.S.D., %) in Table II] were calculated as follows. The surface concentration of each pairing ion was determined from two independent eluates obtained under identical conditions. Three different aliquots (in parallels of five) resulting in concentrations that fall on the linear portion of the respective calibration graph were selected, and the experimental R.S.D. for each aliquot was calculated. The surface concentration values are the averages of the results obtained for the two eluates, and R.S.D. values are the averages of the R.S.D. data obtained for the 2 × 3 aliquots.

HFBA eluted after equilibration (in 40 ml of absolute ethanol) was also determined by the methylene blue method [11], and both the surface concentration and R.S.D. values were in good agreement with the GLC data presented in Table II.

The data points indicated in Figs. 2–11 are each average k'_G values obtained for two independent series of gradients performed under identical conditions.

RESULTS AND DISCUSSION

Although the recommended eluent volume for column equilibration in RP-IPC is 20–25 V_0, data in the literature on equilibration V_0 vary greatly even for the same pairing ion. For example, Ingebretsen *et al.* [13] found that over 40 V_0 were necessary for the equilibration of an isocratic RP-IPC system with a concentration of 0.3 m*M* TBA (as pairing ion) and 1% (v/v) of methanol in the eluent . On the other hand, Werner *et al.* [14] reported 2.5–3 V_0 for re-equilibration in a gradient RP-IPC system using a 2 m*M* concentration of the same pairing ion (TBA) and an acetonitrile gradient ($\Delta\varphi = 0.2$). The same group later reported *ca.* 5.5 V_0 for re-equilibration in an almost identical RP-IPC gradient system [15]. A comparison of the equilibration V_0 values in refs. 13 and 14 underlines the importance of consideration (ii) mentioned earlier.

In order to find guidelines for a judicious selection of "efficient" gradient systems, we examined five pairing ions (of varying alkyl chain length and shape) in two (relatively low and relatively high) concentrations, and gradients with acetonitrile, (buffer) salt and mixtures thereof, as shown in Table I. In this work, gradient system "efficiency" refers to the "speed" of re-equilibration between gradient runs and the ability of the respective gradient to decrease retentions. For the assessment of the latter we used the average retention decrease factor (ARDF), calculated as described under *Procedures*.

A gradient system was regarded as efficient if the number of V_0 necessary for reproducible retentions did not exceed 3, and (at the same time) the ARDF value was equal to or larger than 1.25 for monovalent ionic analytes (an ARDF of 1.25 usually implies that the retention of the compound eluting last was decreased by a factor of *ca.* 1.5, which is mostly the minimum goal when using a gradient system).

It should be emphasized that by "reproducible retentions" we do not necessarily mean that the RP-IPC system is in the state of a complete equilibrium. A quasi-equilibrium, which we denote by "equilibrium" or E, can also result in reproducible

TABLE II

SURFACE CONCENTRATIONS OF PAIRING IONS

Adsorbed by the stationary phase [Nucleosil 5 C_{18} (100 Å), 5 μm, 310 m^2/g, 112 m^2/ml] from the solvents A shown in Table I.

Solvent A	Surface concentration		Solvent A	Surface concentration	
	$mol/m^2 \times 10^8$	R.S.D. (%)		$mol/m^2 \times 10^8$	R.S.D. (%)
1: TFA, 8 m*M*	1.1	20.5	6: SOS, 10 m*M*	37.5	5.7
2: TFA, 200 m*M*	6.2	14.6	7: SDS, 0.5 m*M*	~19.0	–
3: HFBA, 5 m*M*	8.4	10.5	8: SDS, 3 m*M*	54.0	5.2
4: HFBA, 50 m*M*	41.1	8.1	9: TBA, 5 m*M*	35.1	4.1
5: SOS, 1 m*M*	~12.0	–	10: TBA, 60 m*M*	68.8	3.6

gradient retention times under circumstances to be specified later.

In this study, we attempted to relate both the mobile phase and stationary phase pairing ion concentrations to the speed of equilibration. The surface concentrations of the pairing ions (obtained after a 60-min equilibration with the respective solvent A) are shown in Table II. The re-equilibration speeds with the gradient systems studied can be obtained from the graphs of k'_G *vs.* column volumes between runs presented in Figs. 2–11 (see the V_0 values indicated by E). Table III presents the efficiency of the gradient systems shown in Table I in terms of two (combined) criteria: the ARDF value and the number of V_0 of solvent A needed to reach "equilibrium", E, *i.e.*, reproducible retentions.

Inspection of Figs. 2–11 and Table III shows that the speed of re-equilibration increases in the order acetonitrile gradients < mixed gradients < salt gradients, demonstrating that RP-IPC ionic equilib-

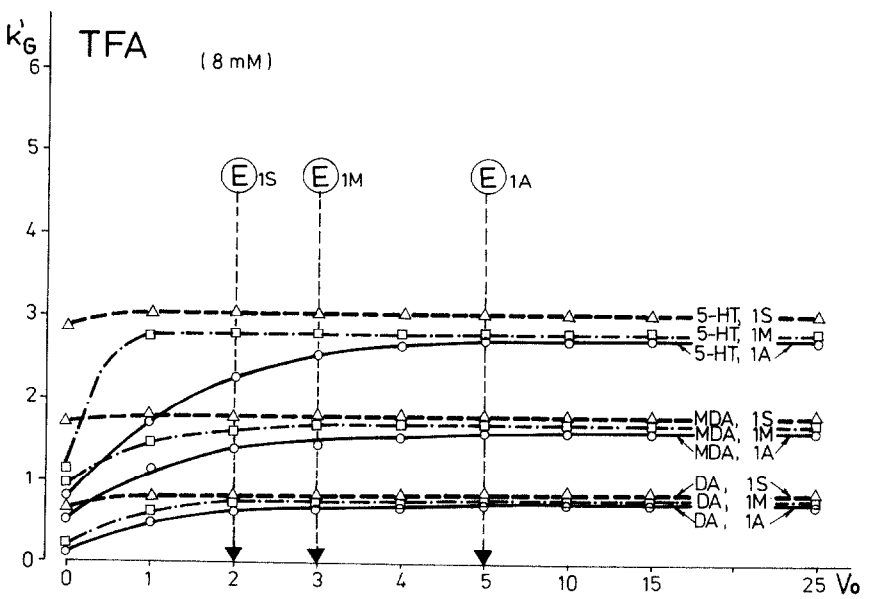

Fig. 2. Plots of k'_G *vs.* column volumes between runs (V_0 of solvent A) with 8 m*M* TFA as pairing ion in the eluents, and acetonitrile (A, ○), salt (S, △) and mixed (M, □) gradients. E indicates the number of V_0 which provide satisfactory equilibration between runs for obtaining reproducible retention times in the respective gradient system. Other conditions as in Table I and Experimental.

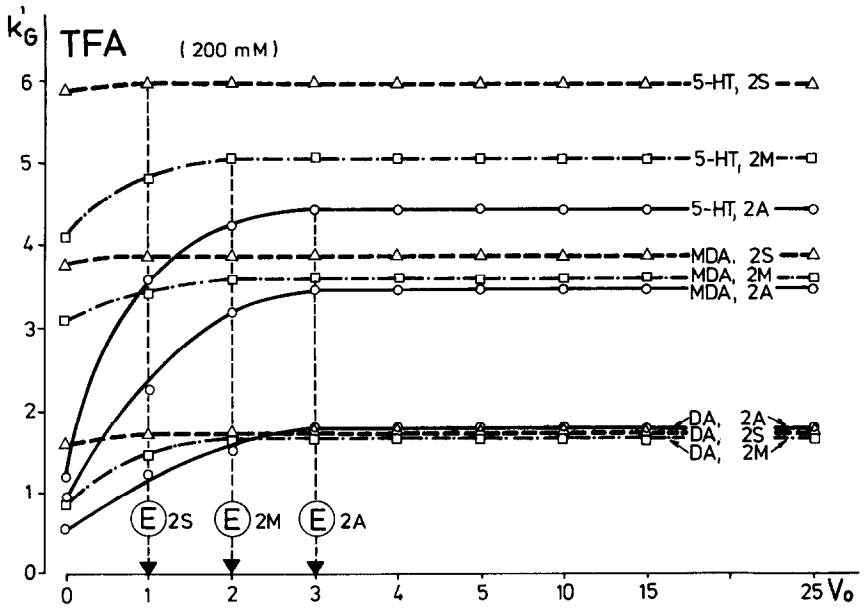

Fig. 3. Plots of k'_G *vs.* column volumes between runs with 200 m*M* TFA as pairing ion in the eluents, and A, S and M gradients. For A, S, M and E, see Fig. 2. Other conditions as in Table I and Experimental.

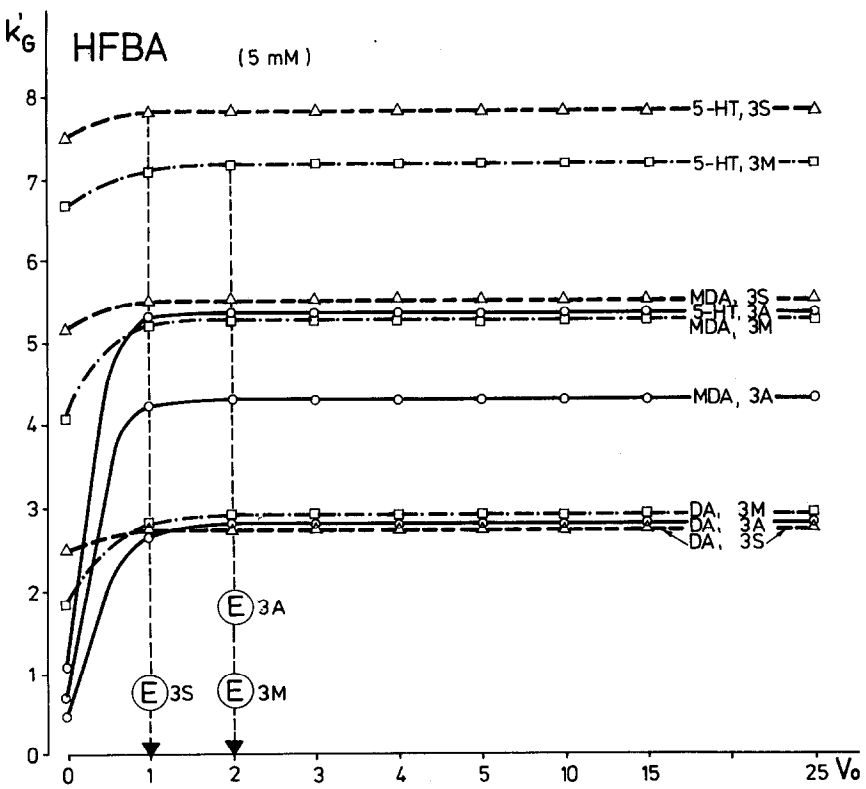

Fig. 4. Plots of k'_G *vs.* column volumes between runs with 5 m*M* HFBA as pairing ion in the eluents, and A, S and M gradients. For A, S, M and E, see Fig. 2. Other conditions as in Table I and Experimental.

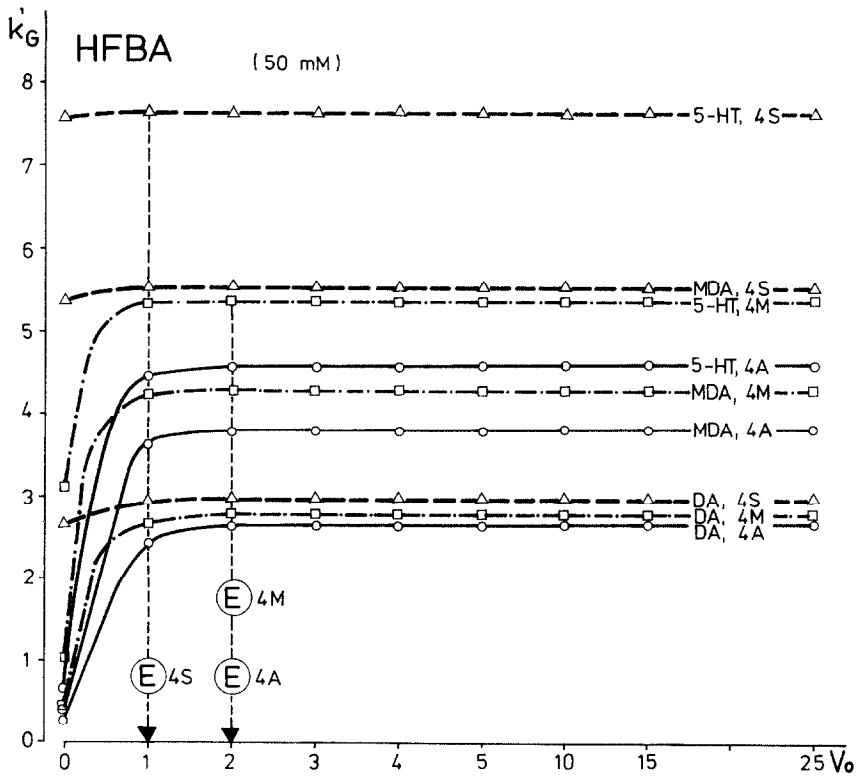

Fig. 5. Plots of k'_G *vs.* column volumes between runs with 50 m*M* HFBA as pairing ion in the eluents, and A, S and M gradients. For A, S, M and E, see Fig. 2. Other conditions as in Table I and Experimental.

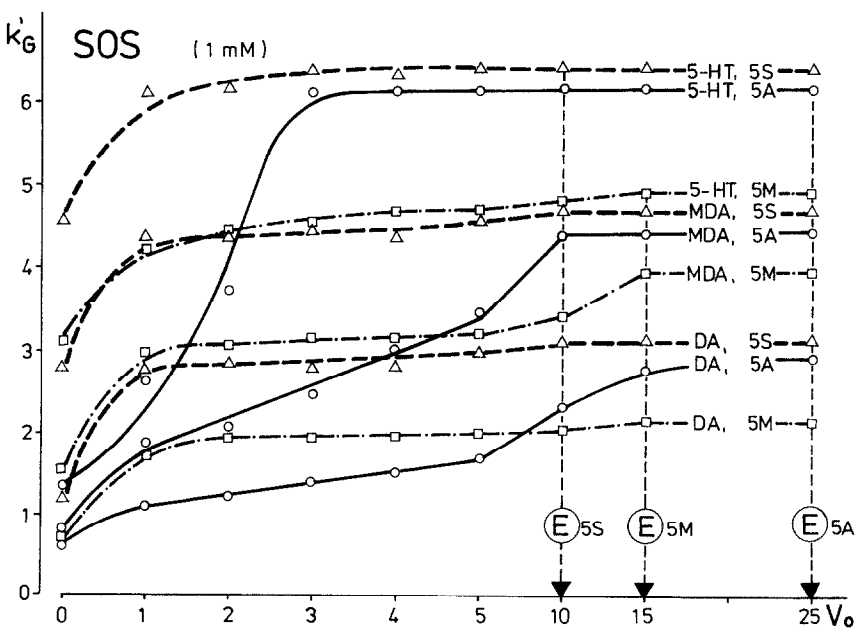

Fig. 6. Plots of k'_G *vs.* column volumes between runs with 1 m*M* SOS as pairing ion in the eluents, and A, S and M gradients. For A, S, M and E, see Fig. 2. Other conditions as in Table I and Experimental.

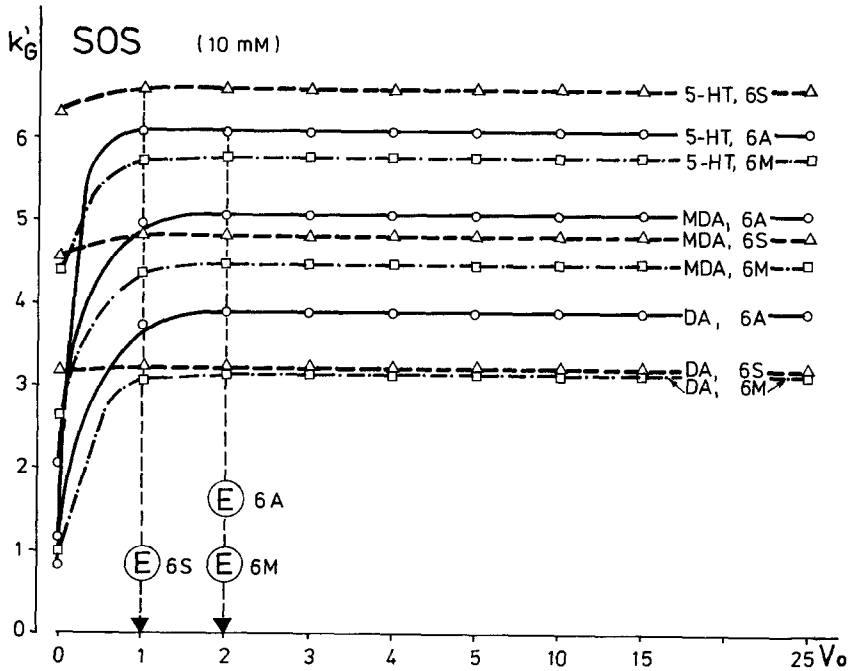

Fig. 7. Plots of k'_G *vs.* column volumes between runs with 10 m*M* SOS as pairing ion in the eluents, and A, S and M gradients. For A, S, M and E, see Fig. 2. Other conditions as in Table I and Experimental.

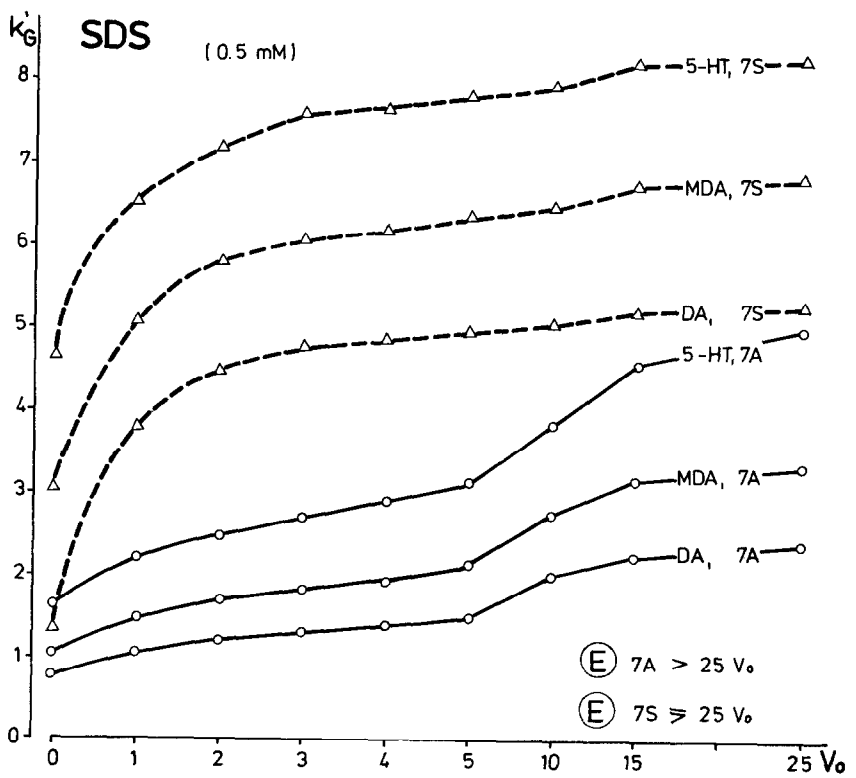

Fig. 8. Plots of k'_G *vs.* column volumes between runs with 0.5 m*M* SDS as pairing ion in the eluents, and A and S gradients. For A, S and E, see Fig. 2. Other conditions as in Table I and Experimental.

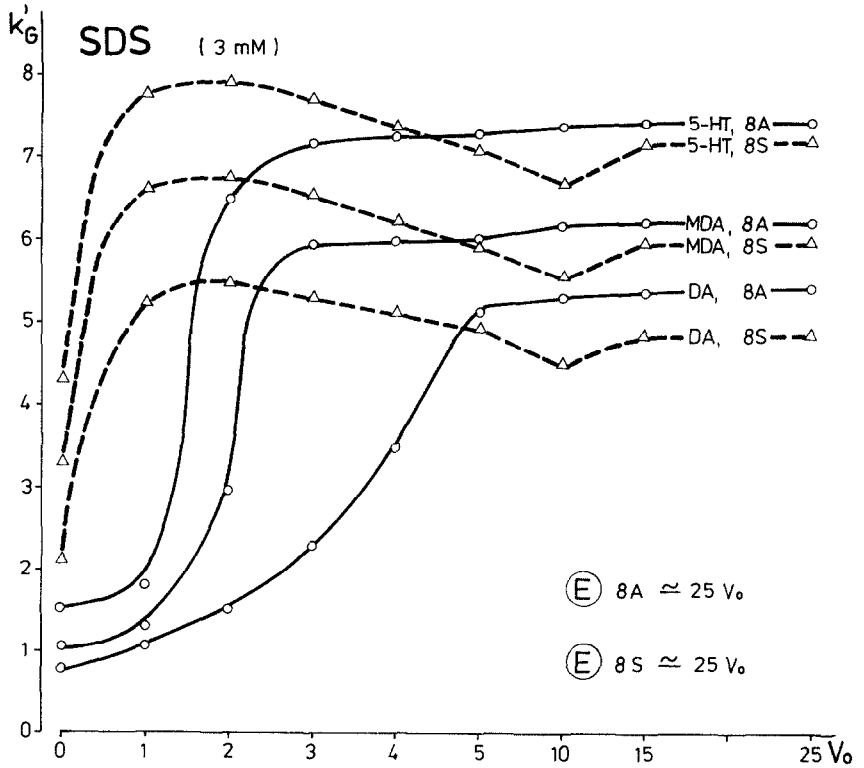

Fig. 9. Plots of k'_G *vs.* column volumes between runs with 3 m*M* SDS as pairing ion in the eluents, and A and S gradients. For A, S and E, see Fig. 2. Other conditions as in Table I and Experimental.

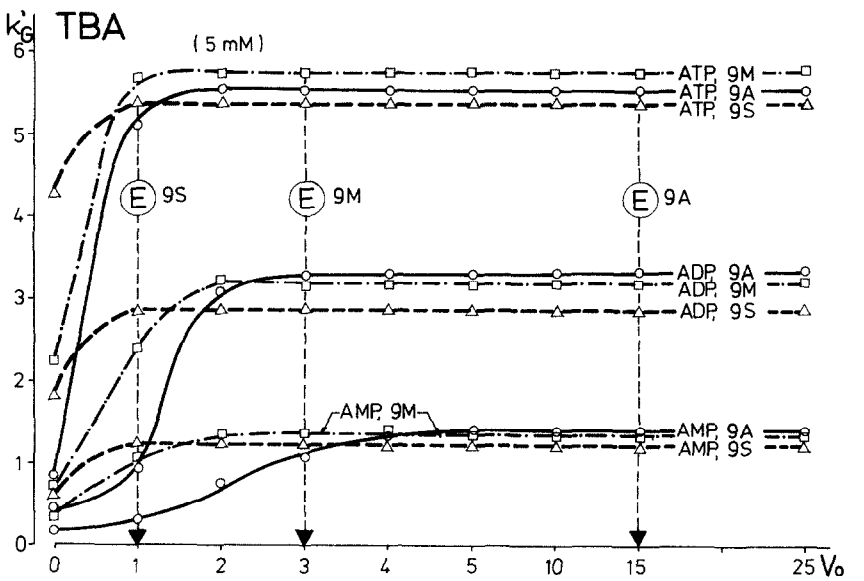

Fig. 10. Plots of k'_G *vs.* column volumes between runs with 5 m*M* TBA as pairing ion in the eluents, and A, S and M gradients. For A, S, M and E, see Fig. 2. Other conditions as in Table I and Experimental.

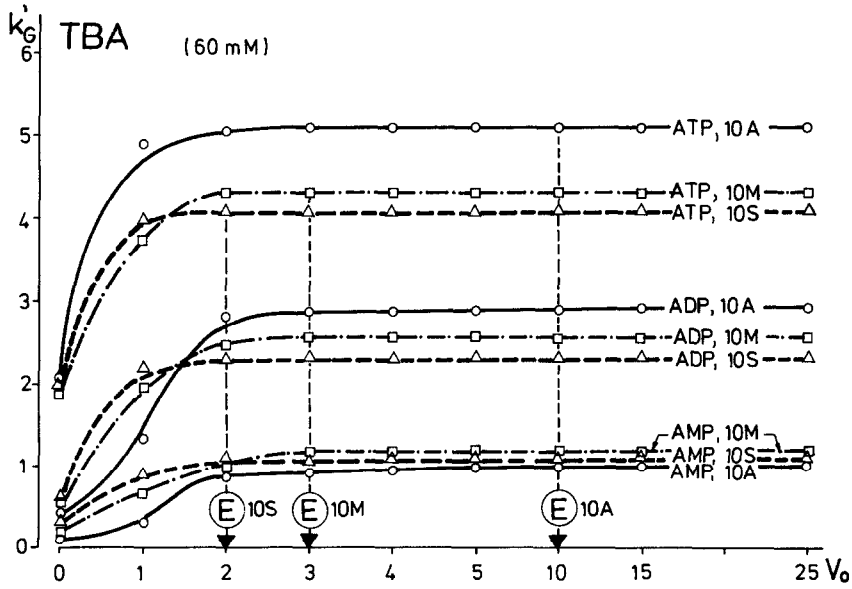

Fig. 11. Plots of k'_G *vs.* column volumes between runs with 60 m*M* TBA as pairing ion in the eluents, and A, S and M gradients. For A, S, M and E, see Fig. 2. Other conditions as in Table I and Experimental.

ria are less disturbed by changes in eluent salt concentrations than by changes in the volume ratio of the organic modifier in the eluent.

Based on the two criteria in Table III, the following salt gradient systems were found to be efficient: systems 3S, 6S, 9S (at the same $\Delta\varphi$, *i.e.*,

TABLE III

"EFFICIENCY" OF THE GRADIENT SYSTEMS

In terms of retention decrease and "speed" of equilibration.

Pairing ion	Gradient notation[c]	ARDF[d]	E[e]	Gradient notation[c]	ARDF[d]	E[e]	Gradient notation[c]	ARDF[d]	E[e]
TFA[a]	1A	1.14	5	1S	1.06	2	1M	1.12	3
	2A	1.21	3	2S	1.03	1	2M	1.15	2
HFBA[a]	3A	1.61	2	3S	1.29	1	3M	1.35	2
	4A	1.64	2	4S	1.13	1	4M	1.45	2
SOS[a]	5A	1.30	25	5S	1.22	10	5M	1.61	15
	6A	1.35	2	6S	1.35	1	6M	1.42	2
SDS[a]	7A	<1.91	>25	7S	<0.98	≥ 25	7M	–	–
	8A	$\simeq 1.51$	$\simeq 25$	8S	$\simeq 1.57$	$\simeq 25$	8M	–	–
TBA[b]	9A	1.42	15	9S	1.53	1	9M	1.46	3
	10A	1.58	10	10S	1.42	2	10M	1.65	3

[a] With monovalent sample cations.
[b] With mono-, di- and trivalent sample anions.
[c] A = Acetonitrile gradient; S = salt gradient; M = mixed gradient.
[d] ARDF = Average retention decrease factor.
[e] E = Number of column volumes (V_0) needed to reach "equilibrium".

85 mM) and 10S (at $\Delta\varphi$ = 135 mM). It is shown in Table II that with systems 3, 6 and 9 the surface concentration of the pairing ion was higher than $8 \cdot 10^{-8}$ mol/m^2, and also that the total counter ion concentration in the starting buffers (the solvent As of these systems, see Table I) did not exceed 25 mM. Hence a satisfactory surface coverage by the pairing ion and, at the same time, a low counter ion concentration in solvent A appear to be two preconditions for an efficient salt gradient system.

Other conclusions drawn from the data in Table III (in combination with Tables I and II and Figs. 2–11) are as follows. The ARDF values in all six gradient systems containing TFA as pairing ion were low. With the salt gradients this is explained by the low surface coverage (system 1S) and/or by the high total counter ion concentration in solvent A (*ca.* 215 mM, system 2S). The ARDF values for the acetonitrile gradients were lower than those of the SOS acetonitrile gradients (actually the lowest among the acetonitrile gradient systems studied), although $\Delta\varphi$ for system 1A was almost the same as for system 5A or 6A (for system 2A it was actually higher). This can be explained by the suggestion put forward by Bidlingmeyer *et al.* [16], *i.e.*, the adsorption of a three-carbon (or shorter) pairing ion (*e.g.*, TFA) from an aqueous buffer on to a hydrophobic surface is less affected by a change in the volume fraction of the organic modifier (methanol or acetonitrile) in the eluent than the adsorption of a pairing ion with a longer alkyl chain. The gradient range in systems 1A and 2A was, therefore, too small for the TFA systems, resulting in the low ARDF values.

For another explanation we must begin by saying that, owing to the very low surface coverage by the pairing ion, TFA systems, in general, are much closer in character to simple reversed-phase high-performance liquid chromatographic systems than to "real" RP-IPC systems in which double-layer sorption effects govern retention [2]. In RP-IPC systems with more hydrophobic pairing ions (and higher surface coverage), organic modifiers are known to decrease the retention of oppositely charged ionic solutes more efficiently than in reversed-phase systems [17] through at least two simultaneous effects: via the decreased hydrophobic adsorption of the solutes and the decreased surface concentration of the pairing ion [18,19]. With TFA systems organic modifiers decrease retentions (almost entirely) by only decreasing the hydrophobic adsorption of the solutes.

The ARDF values with five of the HFBA systems were reasonably good, and the "speed" of equilibration was high in all six gradient systems (see the E values in Table III), demonstrating the excellent kinetic properties of this pairing ion. (With HFBA the surface coverage determined by both the GLC and the methylene blue method was relatively high, but as perfluorinated acids are known to be considerably more hydrophobic than hydrocarbon acids of the same chain length [11], that is easily explained.) The ARDF value of system 4S was low because of the higher than desirable total counter ion concentration (65 mM) in solvent A.

From our point of view, the main gradient "efficiency" problem with RP-IPC systems consisting of eluents (solvent As) with a low concentration of a strongly adsorbed pairing ion (such as SOS, SDS or TBA) and an organic modifier, also in a low concentration, is system stability (see Figs. 6 and 8). TBA and SOS systems can be stable enough when the eluent pairing ion concentration reaches or exceeds 2 mM (see refs. 14 and 15 and Figs. 7 and 10). SDS systems, however, still lack reproducibility of retention at this pairing ion concentration (see Fig. 9).

Although both the surface coverage and the total counter ion concentration in solvent A can be in the right range for salt gradients, the number of re-equilibration V_0s of such RP-IPC systems (with SOS, SDS or TBA concentrations below 2–3 mM in organic modifier-lean solvent As) is unacceptably high (see the E values for systems 5A, 5S, 5M and 7A, 7S, 8A and 8S in Table III). After a 60-min equilibration with solvent A and the determination of the k'_0 values, with such systems the surface coverage continues to increase during the series of salt gradients, resulting in relatively higher k'_G values. This explains the low ARDF value in system 5S and the even lower ARDF value in system 7S in Table III.

Such system stability problems are invariably related to considerations (i) and (ii) in the Introduction, suggesting that for "fast" gradients C_4–C_8-alkyl pairing ions at eluent concentrations larger than 2–3 mM should be preferred, and that pairing ions with slow sorption kinetics, especially those with H-type adsorption isotherms [20] in solvent As

of low organic modifier content, should be avoided.

The pairing ion concentrations in the TBA systems studied were both on the "safe side" of the adsorption isotherm. The 5 m*M* system was tested with mono-, di- and trivalent sample anions and the 60 m*M* system was tested with the same anions and higher gradient ranges ($\Delta\varphi$ = 0.28 and 135 m*M*, respectively).

With system 9, all three ARDF values were acceptable, although lower than expected from ionic solutes of higher charge. The speed of equilibration was excellent with the salt gradient and fairly good with the mixed gradient (systems 9S and 9M, respectively, in Table III). With system 10S, the ARDF value was lower (in spite of the larger $\Delta\varphi$) because the total counter ion concentration in solvent A (75 m*M*) was higher than desirable. The overall "efficiency" of system 10M was, however, better than that expected based on the results for systems 10A and 10S.

In spite of the "safe" pairing ion concentrations, the speed of equilibration with the acetonitrile gradients of the TBA systems was relatively poor (poorer than those reported in refs. 14 and 15 for similar systems and solutes; see Figs. 10, 11 and the E values for systems 9A and 10A in Table III), indicating either that the kinetic properties of TBA are less than ideal for such gradients or (which is logical and expected) that the retention of ionic solutes of higher charge is more sensitive to changes in pairing ion surface concentrations than the retention of monovalent ions.

To summarize, it appears from the results obtained in this study that the throughput of RP-IPC gradient elution systems can be improved considerably by selecting chromatographic conditions under which the volume of solvent A used for re-equilibration between gradient runs can be reduced to 1–3 column volumes. Chromatographic conditions for such "efficient" RP-IPC gradient systems include: (1) using C_4–C_8-alkyl pairing ions at eluent concentrations of 3–10 m*M* (depending on the nature of the pairing ion and the organic modifier content of solvent A; solvent A itself should be a stable isocratic system); and (2) keeping the change in pairing ion surface concentrations to the necessary minimum during a gradient run, by using salt gradients (for the separation of ionic compounds) or mixed gradients (for the separation of ionic and neutral compounds), instead of "pure" acetonitrile gradients.

For "efficient" salt gradients and mixed gradients two (additional) preconditions should be met: (a) the adsorbent surface coverage by the pairing ion from solvent A should be larger than (6–8) · 10^{-8} mol/m^2 [with C_4–C_8-alkyl pairing ions this is easily achieved by the eluent concentrations specified above if the acetonitrile or methanol content of solvent A does not exceed 5–15% (v/v)]; and (b) the total counter ion concentration in solvent A should be kept below 20–25 m*M*, just enough for a satisfactory buffering capacity.

For "efficient" acetonitrile gradients there is special emphasis on the kinetic properties of the pairing ion used. For cationic and neutral analytes HFBA appears to be a very good pairing ion candidate. Theoretically, TFA (used with a relatively larger gradient range) should also exhibit good kinetic properties in such gradients, but we have difficulty in explaining the relatively poor E value with system 1A in Table III (see also Fig. 2).

For satisfactory reproducibility of retention with any of the RP-IPC gradient systems studied, it was essential to reset the system to solvent A always at the same time point at the end of a gradient.

When considering these guidelines for the selection of efficient RP-IPC gradient systems, it should be kept in mind that "efficiency" is meant here only with respect to the "speed" of re-equilibration and for the ability of the respective gradient to reduce retentions. In terms of system selectivity, for example, there may be substantial differences in the chromatographic patterns obtained (for the same group of analytes) by a salt gradient, a mixed gradient or an acetonitrile gradient separation. These gradients may not result in equivalent separations and may not be equally efficient in terms of selectivity.

The steps recommended here for increasing the throughput of RP-IPC gradient separations do not solve the general problem of slow equilibration in ion-pair gradient elution. By adopting the conditions recommended for salt (and mixed) gradients, for instance, one actually strengthens the chances for dynamic ion-exchange in the diffuse part of the electrical double layer (as compared with the chances of surface adsorption) to determine retentions, as can be seen in Fig. 3 in ref. 2. Under the conditions we specified for RP-IPC salt gradients, retention is dominantly determined by ion-exchange processes and concomitant selectivities.

From the foregoing, it is also clear that, because of the very low surface coverage attainable by TFA as pairing ion and, as a result, the very weak presence of ion-exchange features in such RP-IPC systems, this study has little to offer for increasing, *e.g.*, the throughput of peptide separation systems with TFA as pairing ion.

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