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ROLE OF THE CHARGE NUMBER OF THE COUNTER-IONIC CONSTITUENT IN THE SEPARATION OF ANIONS BY ISOTACHOPHORESIS*

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SUMMARY

The influence of the different charge numbers of the ionic forms of the buffering counter-ionic constituents on the effective mobilities of anions at pH 6.0 was investigated. It is shown that a proper choice of the charge number of the counter-ionic constituent can be used as an effective tool in optimization of the operating conditions in the separation of anions by isotachophoresis. The successful separation of a group of anions at pH 6.0 using 1,3-bis[tris(hydroxymethyl)methylamino]propane as the buffering counter constituent in the leading electrolyte (which could not be performed at this pH when other constituents were used for this purpose) illustrates the practical possibilities of this approach. Ca^{2+} (a complex-forming cation) and doubly protonated diaminopropane (a non-complexing cation) were used as co-counterions in the leading electrolytes to show the different natures of their interactions with the same group of anions.

INTRODUCTION

There are several means by which the effective mobilities of ionic constituents can be affected in a desired way and, consequently, their isotachophoretic separations effected[1]. Of these, the pH dependences of the effective mobilities are mostly used, and in some instances complex formation is a good alternative[2,3]. When the charge numbers of the components to be separated differ, their effective mobilities exhibit

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different concentration dependences. This property can also be of practical use in isotachophoresis[1].

Supposing that no complex formation occurs, in the above concentration dependences both electrophoretic and relaxation effects[1,4-7] and ion pairing[4-7] are involved. In the terms that are used to correct conductivities or mobilities for electrophoretic and relaxation retardations, in addition to concentrations of the ions and parameters describing the properties of the solvent, ionic charges are also involved[1,4-7]. The electrophoretic and relaxation retardations are long-range effects and should be completely independent of any short-range parameters such as ionic dimensions[6]. On the other hand, ion pairing should include all short-range effects[6] (ion–ion and ion–solvent interactions).

Everaerts et al.[1] have shown that corrections of the calculated conductivities of the zones of some divalent cations and anions for the electrophoretic and relaxation effects were sufficient to obtain theoretically predicted dependences of the thermal step heights on isotachopherograms on the resistivities of the zones. This means that for the counter-ionic constituents used and the ionic constituents investigated ion pairing is negligible. Moreover, it is known from conductivity measurements[8] that strong 2–2 electrolytes which have separated their charge-carrying groups by an inert framework can serve as good model constituents with no ion pairing concentrations up to at least 5 mM.

The above facts provide the possibility of a qualitative interpretation of the results presented in this work. For greater detail, an extensive literature dealing with many aspects of the theory of electrolytic conductance is available (e.g., refs. 5–7).

The aim of this work was to show how different counter-ionic constituents buffering at the same pH, while differing in the charge numbers of their ionic forms at this pH, influence the effective mobilities of anions. It will be shown that a proper choice of the charge number of the counter-constituent gives some practical possibilities for the separation of anions by isotachophoresis. At pH 6.0 doubly protonated diaminopropane[7] (a non-complexing cation) and Ca^{2+} (a complex-forming cation) used at the same concentrations in the leading electrolytes can give an insight into the nature of the interactions that are responsible for the retardations of anion in these instances. The former represents mainly retardation due to the electrophoretic and relaxation effects, while the latter introduces a combined effect of both the long-range and short-range interactions.

As previously no attention had been paid to the subject dealt with in this paper, an attempt is made here to explain some of the phenomena observed.

EXPERIMENTAL

An instrument for isotachophoresis similar to that described by Everaerts et al.[1] was used, provided with a conductivity detection cell designed by Stankoviansky et al.[9]. A fluorinated ethylene–propylene copolymer (FEP) capillary tube of I.D. 0.3 mm was used.

Chemicals were of pro analisi purity and some of them were purified by conventional methods.

Histidine (HIS), 1,3-bis[tris(hydroxymethyl)methylamino]propane (bis-tris-propane or BTP) and 2-[N-morpholino]ethanesulphonic acid (MES) were bought

from Sigma (St. Louis, MO, U.S.A.). 1,2-Dimorpholinylethane (DME) was obtained from K & K (Plainview, NY, U.S.A.) and lysine (LYS) and 1,3-diaminopropane (DAP) from Fluka (Buchs, Switzerland). The other chemicals were supplied by Lachema (Brno, Czechoslovakia).

As additives to the leading electrolytes[1], Mowiol 8-88 (Hoechst, Frankfurt, G.F.R.) or hydroxyethylcellulose (Polysciences, Warrington, PA, U.S.A.) were used at 0.05% and 0.2% concentrations, respectively.

RESULTS AND DISCUSSION

The operating systems used are listed in Table I. The concentration of the leading anion (chloride, 0.01 M) and the pH of the leading electrolytes (6.0) were kept constant. Counter-ionic constituents buffering at pH 6.0 and differing in the charge numbers of their ionic forms were varied.

TABLE I

OPERATING SYSTEMS

Additives to the leading electrolytes: Mowiol 8-88 or hydroxyethylcellulose. Terminating anions: 0.005 M MES or 0.005 M capronate. For the abbreviations used, see Experimental.

Parameter	System No.										
	1	2	3	4	5	6	7	8	9	10	11
Leading anion	Cl⁻	Cl⁻	Cl⁻	Cl⁻	Cl⁻	Cl⁻	Cl⁻	Cl⁻	Cl⁻	Cl⁻	Cl⁻
Concentration (M)	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Counter ion	HIS	HIS	HIS	HIS	DME	BTP	DME	DME	DME	DME	HIS
Co-counter ion	—	DAP	DAP	DAP	—	—	LYS	LYS	HIS	DAP	Ca^{2+}
Concentration (M)	—	0.001	0.002	0.003	—	—	0.002	0.006	0.006	0.002	0.002

Some of the operating systems were used for several reasons:

(1) to explain the observed changes in the effective mobilities of anions for different buffering counter-constituents by using the results obtained in other systems;

(2) to compare retarding efficiencies of co-counter ions carrying the same or different charges;

(3) to compare the effective mobilities of anions for the co-counterions carrying the same charges while differing in the interactions involved (*e.g.*, long-range effects and complex formation);

(4) to investigate dependences of the effective mobilities of anions carrying different charges on the concentration of a co-counterion in the leading electrolyte.

It is assumed in the following discussion that only negligible differences in the pH of the zones exist when different buffering counter constituents (small differences in their pK values) are used at the same pH and/or the buffering capacities of the leading electrolytes containing non-buffering co-counter ions are sufficient. These assumptions are supported by several facts: at pH 6.0 the anions studied behave more or less like strong electrolytes; no phenomena known to occur when a leading electrolyte with unsufficient buffering is used[1] were observed (the anode compartment of the separation unit was filled with an equimolar solution of histidine and histidine

hydrochloride to prevent the protons formed in this compartment from entering the separation compartment); very good reproducibility of the step heights of the zones on the isotachopherograms was found for all of the operating systems.

As the constituents studied are almost fully dissociated at pH 6.0, their ionic mobilities and in part also their association equilibria (ion pairing) are affected when the counter constituents are changed.
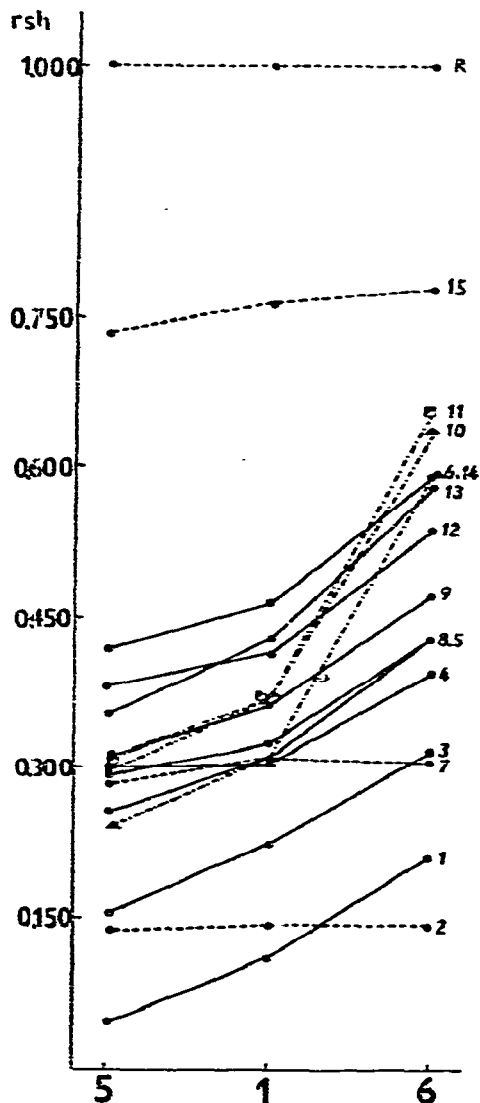

Fig. 1. Relative step heights (rsh) of the anionic constituents relative to trichloroacetate for different counter-ionic constituents buffering at pH 6.0. 1 = Oxalate; 2 = chlorate; 3 = tartronate; 4 = fumarate; 5 = malonate; 6 = *trans*-aconitate; 7 = formate; 8 = tartrate; 9 = malate; 10 = citrate; 11 = isocitrate; 12 = pyrazole-3,5-dicarboxylate; 13 = pyrazine-2,3-dicarboxylate; 14 = maleate; 15 = acetate; R = trichloroacetate. For compositions of the operating systems, see Table I. - - -, Monovalent; -.-.-, trivalent; ——, divalent.

The step heights of the anions on the isotachopherograms relative to trichloro-acetate (see ref. 1, p. 307) are used for the evaluation of the results.

The relative step heights of the anions for different counterconstituents buffering at pH 6.0 are given in Fig. 1. In some instances, as can be seen from the data, different orders of some anions in the steady state can be expected on changing one counter constituent for another that differs in the charge numbers of its ionic forms. The isotachopherograms in Fig. 2 clearly illustrate this fact. A complete separation of a group of anions was achieved using a leading electrolyte containing BTP as the counter constituent. Histidine and DME did not effect this separation at the same pH.
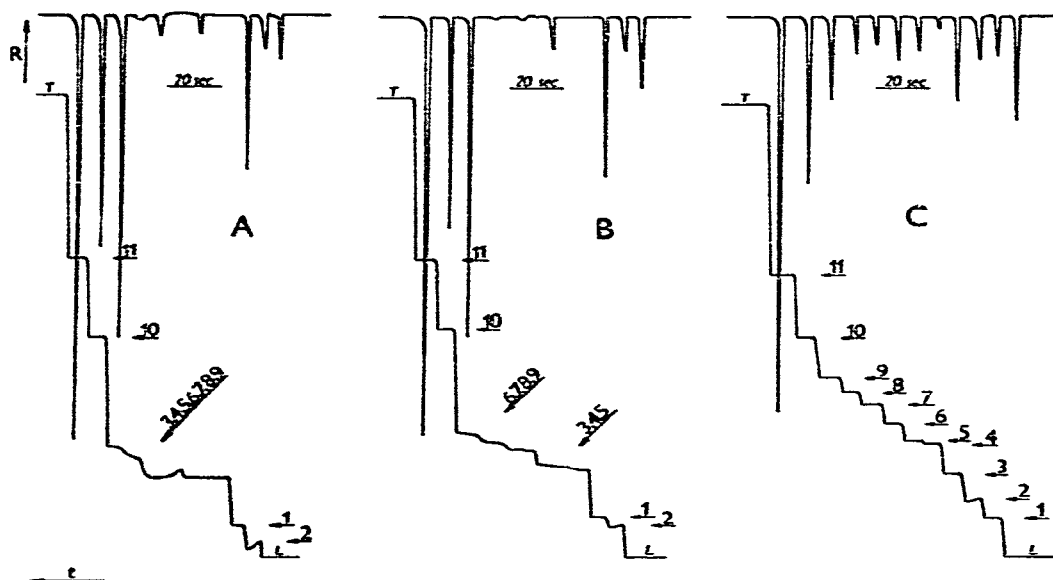

Fig. 2. Isotachopherograms for the separation of a group of anions at pH 6.0. Different counter-ionic constituent buffering at this pH were used in the leading electrolytes: A, DME (system 5); B, histidine (system 1); C, BTP (system 6). A 1-μl volume of the sample (1 = chlorate; 2 = oxalate; 3 = formate; 4 = fumarate; 5 = tartrate; 6 = malate; 7 = pyrazole-3,5-dicarboxylate; 8 = pyrazine-2,3-dicarboxylate; 9 = citrate; 10 = acetate) was injected in all instances. L = Leading anion (chloride); T = terminating anion (capronate); R = increasing resistance; t = time. The driving current was 50 μA.

The above discussion implies that the separation effect of BTP at pH 6.0 can be ascribed to the higher positive charges of its ionic forms (doubly positively charged acidic form and a singly positively charged basic form) relative to those of histidine and DME. In other words, retardations of the separands due to the electrophoretic and relaxation effects are different in the operating systems used. Among the parameters that describe these effects (e.g., ref. 1, p. 36), the ionic charges of the counter constituents were changed in the operating systems, so that their influence on the effective mobilities is of major importance. The extent to which ion pairing contributes to the retardations of mono- and divalent anions seems marginal with respect to the findings of Atkinson et al.[8]. However, for trivalent anions and/or for counter

constituents distributed into ionic forms carrying higher positive charges than those used in this work, ion pairing could play a dominant role[10].

Figs. 1 and 2 indicate smaller differences in the effective mobilities of the anions for systems 1 and 2. Histidine and DME were used as the buffering counter-ionic constituents. Again, the charge numbers of their ionic forms can explain the differences observed. Histidine (at pH 6.0) is distributed into a doubly positively and a singly negatively charged acidic form and a singly positively and a singly negatively charged basic form. On the other hand the acidic form of DME at this pH has only a single positive charge, while its basic form is uncharged. If it is assumed that only
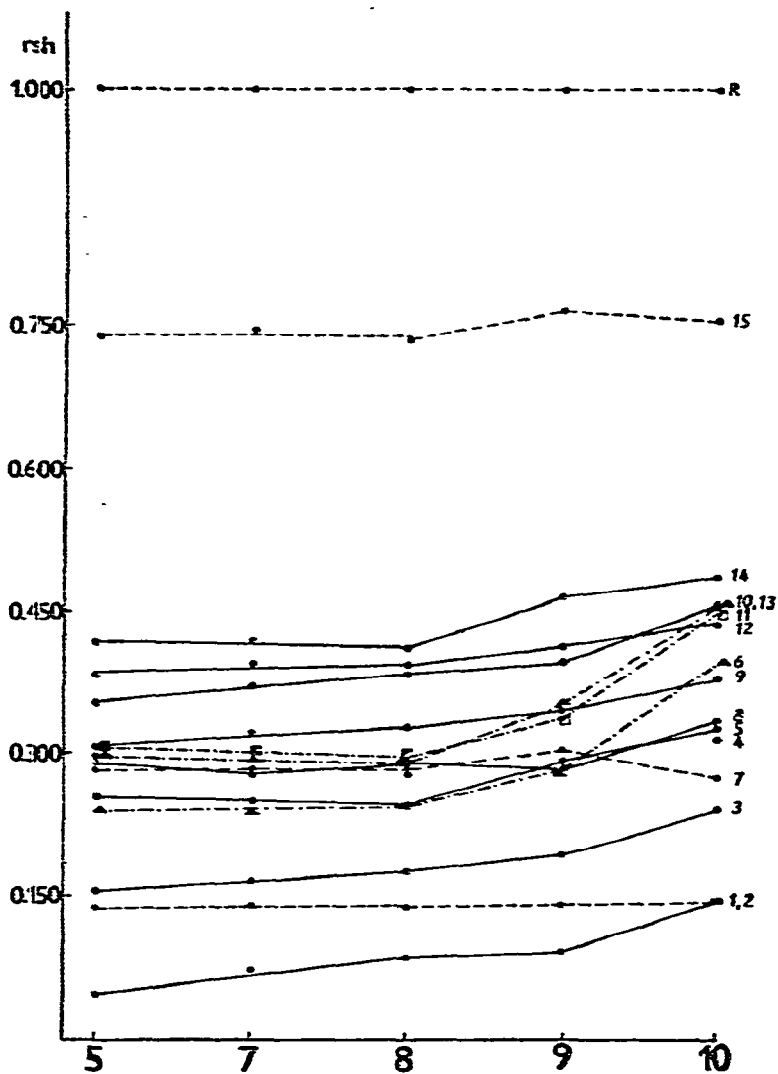

Fig. 3. Relative step heights of the anions at pH 6.0 for different co-counter ions. Information on the operating systems is given in Table I. For numbering of constituents, see Fig. 1. – – –, Monovalent; ——, divalent; –.–.–, trivalent.

the long-range effects are responsible for the different effective mobilities of the anions in these operating systems, histidine must exhibit a higher positive net charge than DME.

Further, the effective mobilities of the anions were measured in systems 7–10 to compare the effects of the same charge type of zwitterionic constituents and to relate them to the results obtained for the system with no co-counter ion (system 5) and to those when 2 mM DAP^{2+} was used (system 10). In these experiments histidine
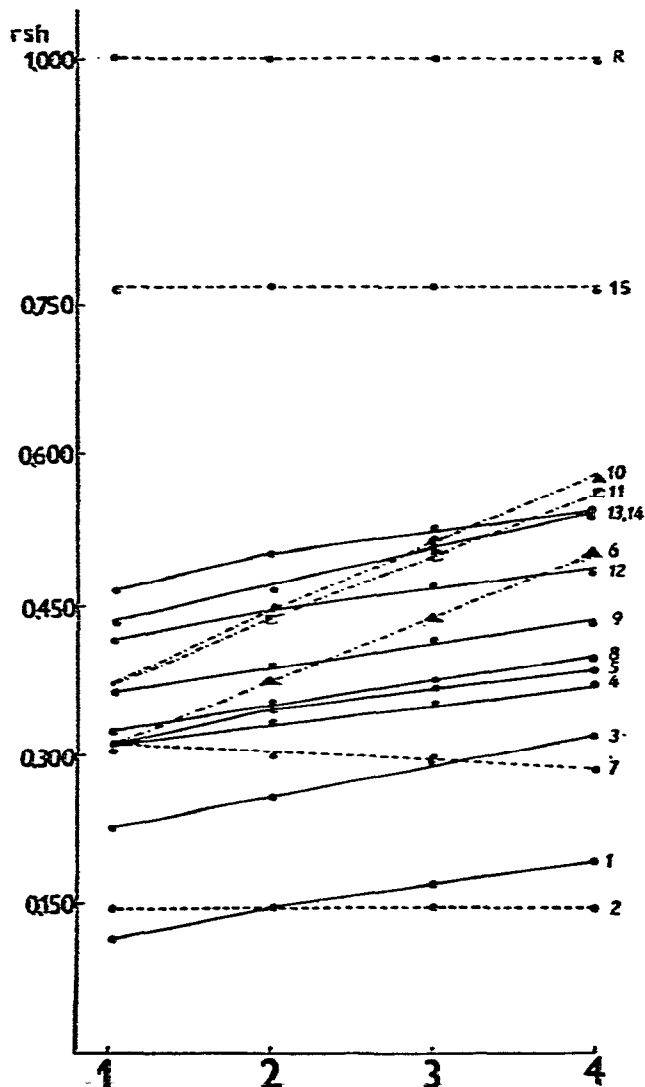

Fig. 4. Relative step heights (rsh) of the anions for different concentrations of DAP^{2+} in the leading electrolytes. Information on the operating systems is given in Table I. For numbering of constituents, see Fig. 1. – – – , Monovalent; ——, divalent; –·–·–, trivalent.

and lysine were used as the co-counter ions. The relative step heights of the anions are given in Fig. 3. For some of them differences in their effective mobilities for the leading electrolytes containing histidine and lysine at the same concentrations can be seen. In general, histidine is a more retarding constituent than lysine, which is unexpected because the opposite would be observed with respect to the above results as lysine at pH 6.0 is completely in the form of a doubly positively charged and a singly negatively charged ion, whereas histidine is only half in this form (see above). A reasonable explanation of these results could only involve higher intramolecular shielding of the opposite charges in the molecules of lysine relative to histidine, rather than a higher intermolecular association of the anions with histidine. The former also explains the smaller than expected long-range effects for lysine at pH 6.0. Nevertheless, further research is necessary in order to obtain a full explanation of these observations.

To show the influence of the concentration of a doubly positively charged co-counter ion on the effective mobilities of the anions, a series of experiments in systems 1–4 were carried out. The results (Fig. 4) show that retardations of the anions are proportional to their charges. In this way it seems possible to estimate the charge numbers of the separated components to allow their identification from a universal detector response[11].

Some anions can be separated by isotachophoresis using labile complex equilibria[2,3]. The isotachopherograms in Fig. 5 show the differences in the order of the components in the steady state when a non-complexing cation (DAP^{2+}) in the
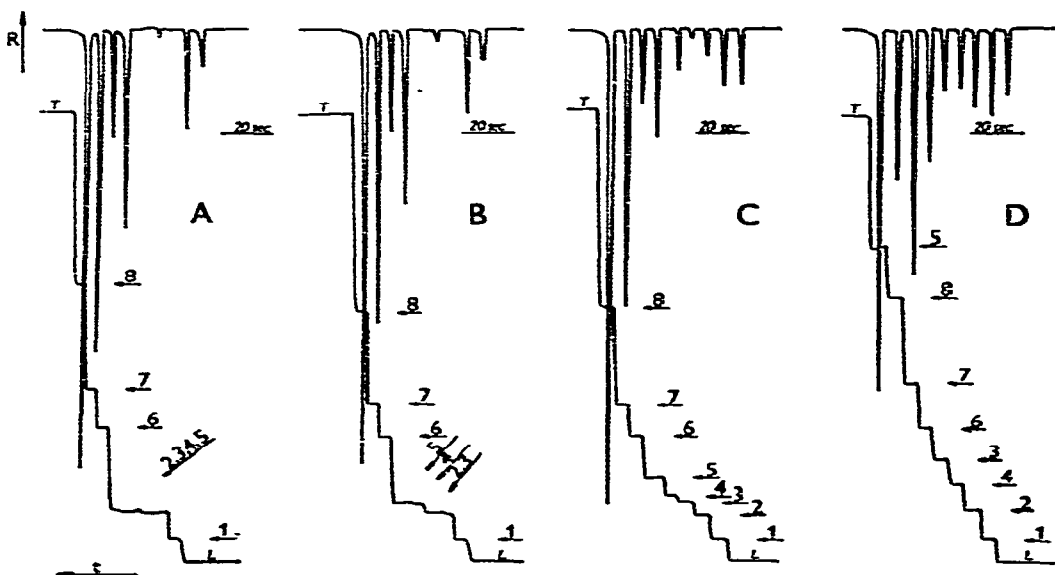

Fig. 5. Isotachopherograms for the separation of a group of anions at pH 6.0. Effects of a complex-forming cation and a non-complexing cation when used as co-counter ions in the leading electrolytes. A, DME (system 5); B, histidine (system 1); C, 2 mM DAP^{2+} (system 3); D, 2 mM Ca^{2+} (system 11). A 1-μl volume of the sample (1 = chlorate; 2 = formate; 3 = tartrate; 4 = α-ketoglutarate; 5 = citrate; 6 = acetate; 7 = lactate; 8 = capronate) was injected in all instances. L = Leading anion (chloride), T = terminating anion (MES), R = increasing resistance, T = time. The current was stabilized at 50 μA.

leading electrolyte was replaced with a complex-forming cation (Ca^{2+}) at the same concentration. This indicates the different natures of the interactions that are responsible for the separations in these instances: a more specific retardation of the complex-forming cation on the one hand and a less specific retardation due mainly to the electrophoretic and relaxation effects on the other. For this group of anions we can again see a difference in their effective mobilities when histidine and DME were used as counter constituents.

At a lower pH of the leading electrolyte (3.0–5.0) a similar behaviour of anionic constituents was found when buffering counter constituents differing in their charge numbers were used. Moreover, some phenomena that disturbed the separations of some anions were observed when counter constituents carrying higher positive charges at these pH values were used. Further research on this aspect is being carried out in order to obtain an explanation of the phenomena observed.

CONCLUSIONS

The charge numbers of the ionic forms of the buffering counter-ionic constituents play an important role in separations of anions by isotachophoresis. Therefore, the counter constituents should not only be chosen for their buffering properties at a particular pH but also the charge numbers of their ionic forms should be taken into account.

A proper choice of the buffering counter-ionic constituent can increase the number of components resolved at a given pH or at least a higher resolution rate[12] can be achieved in this way. Obviously, when an attempt is made to reproduce published results the above facts must be borne in mind, otherwise contradictory results could be obtained.

Information concerning of the charges of the separated anions at a given pH can be obtained when the results obtained in at least two operating systems (differing, for example, in the charge numbers of the ionic forms of the counter-ionic constituents used) are compared.

The nature of the separation effects depends on both the charge numbers and the structural factors of the ionic constituents involved in the ionic interactions.

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