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Review

Enhanced selectivity for capillary zone electrophoresis using ion-pair agents

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Abstract

Capillary zone electrophoresis (CZE) is unique in its simplicity and versatility for liquid separations, yet selectivity for solutes with similar charge and hydrated radius is often lacking. Conventional reversed-phase liquid chromatography (RP-LC) ion-pairing agents, ionic polymers, Group I and II metal cations and dicarboxylic acids have all been effectively used over the past decade to enhance CZE resolution through a combination of complexing effects. This review summarizes the fundamental theory of ion–ion interaction as applied to CZE separations and discusses several of the most important and diverse applications of selectivity enhancement that utilize ion-pair equilibria. In addition, the effect of ion-pair additives on electroosmotic flow and examples where changes in analyte selectivity are unrelated to ion–ion interaction are discussed.

Keywords: Selectivity; Ion-pairing reagents; Reviews

Contents

1. Introduction

It is not an overstatement to suggest that capillary electrophoresis (CE) has become recognized as the most versatile analytical separations tool currently

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available to the greater scientific community. No other single technique has been used for such a diverse range of applications including separations of simple and complex inorganic ions, conventional organic solutes, carbohydrates, proteins, DNA and synthetic polymers. Impressively, excellent chromatographic efficiency, high throughput and minimal sample volume requirements are preserved in each of the various modes of CE that have thus far been developed, and unlike conventional HPLC applications, all may be carried out using the same separation column. Yet in spite of the ever-expanding list of applications, in its most fundamental form, capillary zone electrophoresis (CZE) is able to achieve separation strictly on the basis of solute charge/size ratio which in many cases provides relatively little inherent selectivity. The ingenuity and creativity of CZE practitioners has expedited the development of more useful separation strategies, however, that take advantage of selective complexation, intercalation, partitioning and physical entanglement. In this review we will examine the contributions of several groups that have resulted in methods for enhancing selectivity in CZE through (1) ion-pair formation, and/or (2) the use of classical chromatographic ''ion-pair'' agents. (It is important to differentiate between these two cases as we shall see in later discussion.) It is useful to point out that a significant effort utilizing ion-pairing interactions for selectivity enhancement has also been conducted using capillary isotachophoresis (cITP) [1–7] and micellar electrokinetic chromatography (MEKC) [8–10], although this work will not be separately considered here. The work referred to in the following examples is intended to be representative of the numerous types of free-zone systems that have benefited from the use of ion-pairing additives, and the relevant theoretical framework that has been developed to describe their use.

2. Theory

In considering the potential benefits of using ion-pairing agents to enhance selectivity for CZE, it is useful to first describe the equilibrium developed between a solute (S) and the ion-pair additive (A) contained in the electrophoretic buffer. As shown in Eq. (1), this equilibrium is assumed to be rapid and reversible in nature:

$$S + A \overset{K_{ip}}{\leftrightarrow} SA \quad K_{ip} = [SA]/[S][A] \tag{1}$$

As pointed out by Weldon et al. [11], adding an ion-pair complexing additive to the sample without incorporating it into the electrophoretic run buffer provides no real advantage in selectivity, since the analyte and additive (of opposite charge) will quickly separate as soon as the voltage is applied. When used as a component of the run buffer, however, the solute will on average spend part of the separation period associated with the additive and remaining separation time uncomplexed, depending on the relative magnitude of K_{ip}.

Terabe and Isemura [12,13] published a comprehensive discussion of the theory of ion-pair complexation as applied to CZE in 1990. Although these authors used the term ''ion-exchange electrokinetic chromatography'' (EKC) to describe the complexation occurring between (+) charged polymeric buffer additives and anionic solutes, the equations developed are appropriate to our present discussion of ion-pair formation. Using definitions developed by these authors [12], if υ_{ep}(free) and υ_{ep}(pi) represent the velocity of the free analyte ion and pairing ion, respectively, υ_{eo} represents the electroosmotic flow (EOF) velocity and R is the fraction of free ion not interacting (i.e., complexed) with the pairing ion, then the total velocity of the solute, υ_s, is given by:

$$\upsilon_s = \upsilon_{eo} + R\upsilon_{ep}(\text{free}) + (1 - R)\upsilon_{ep}(\text{pi}) \tag{2}$$

Differences in the total velocity ($\Delta\upsilon_s$) of any two solutes (1 and 2) (assuming similar mobilities in the absence of additive) will be maximized when the magnitudes of the formation constants of each solute with the additive (K_{ip1} and K_{ip2}) are substantially different, and when the difference in the electrophoretic velocity of free vs. complexed analyte [υ_{ep}(free) − υ_{ep}(pi)] is significant as given by Eq. (3):

$$\Delta\upsilon_s = \frac{(K_{ip2} - K_{ip1})[A]\left[\upsilon_{ep}(\text{free}) - \upsilon_{ep}(\text{pi})\right]}{(1 + K_{ip1}[A])(1 + K_{ip2}[A])} \tag{3}$$

One potential disadvantage of ion-pair complex-

ation is that deleterious selectivity effects will be observed for two solutes that exhibit inherently different mobilities in the absence of complexing agent (e.g., $\upsilon_2 > \upsilon_1$), but for which the relative magnitudes of the equilibrium constants favor a reduction in separation distance (e.g., $K_{ip2} > K_{ip1}$). As an example, Fig. 1 demonstrates the change in selectivity that is observed when butanesulfonic acid is added as an ion-pair agent to sodium phosphate buffer for the separation of four neurotransmitters [11]. It is noted that while that electrophoretic selectivity of serotonin relative to dopamine increases with the addition of the ion-pair agent, selectivity between the second critical pair, serotonin vs. norepinephrine, decreases. Fig. 2 provides a graphical representation of the effect of ion-pair agent concentration on selectivity for these two critical pairs, which closely correlates to plots of $\Delta\upsilon_s$ vs. % ion-pairing polymer derived by Terabe and Isemura in their earlier work [13].

While it initially appears that complexation between ion-pair additive and solute may be mathematically treated in a straightforward manner, additional effects often arise in practical use that complicate the predictability of solute/additive behavior. For example, EOF is usually substantially reduced by the use of ion-pair modifiers, leading to significant changes in resolution that may not be directly associated with an actual change in analyte selectivity. For this reason, it is necessary to measure EOF effects independently using a non-complexed marker when numerical evaluation of selectivity is desired [11,13]. Further, additional solute–additive interaction associated with hydrophobic complexation frequently complicates efforts to ascertain the selectivity effects strictly due to ion-pair interaction. It is therefore essential to recognize that in some cases, the use of so-called ion-pair agents in the electrophoretic buffer may yield changes in analyte resolution that are not related to ion-pair complexation. Nonetheless, method development with such agents often results in substantial improvements in CZE selectivity for solute classes including both small and macromolecules.

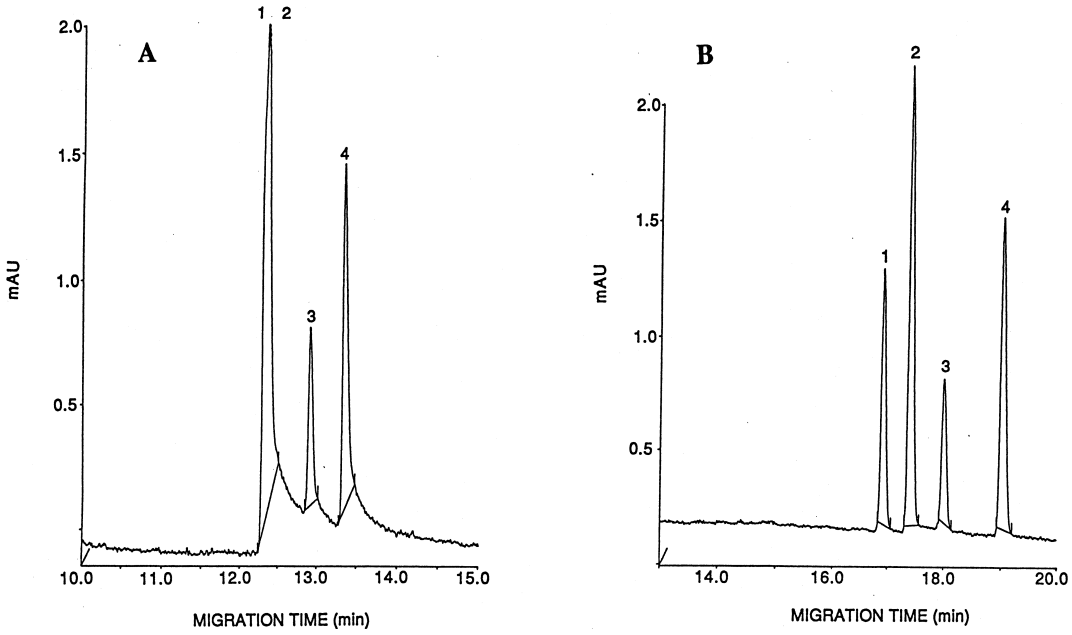

Fig. 1. Separation of neurotransmitters in 20 m*M* phosphate, pH 2.5, (1) dopamine; (2) serotonin; (3) norepinephrine; (4) epinephrine. (A) Buffer only; (B) buffer + 100 m*M* butanesulfonic acid. (Adapted from Ref. [11]).

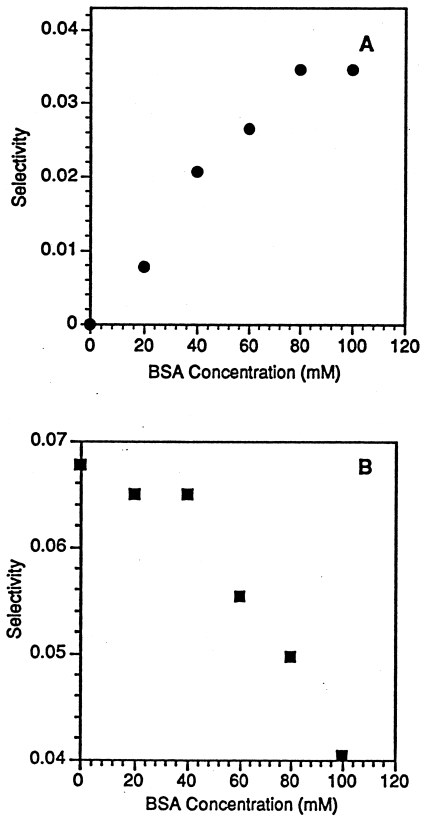

Fig. 2. Selectivity plotted as a function of BSA concentration for (A) dopamine vs. serotonin; (B) serotonin vs. norepinephrine based on Fig. 1 (Reprinted from Ref. [11]).

3. Applications

3.1. Conventional HPLC ion-pair agents

Among the early anecdotal reports of the use of conventional RP-LC ion-pair agents for improving CZE selectivity were accounts of *n*-alkylsulfonic acids used for enhancing peptide digest separations. Although CZE and RP-LC techniques for peptide mapping provide orthogonal separation mechanisms, resolution in the CZE mode is often limited due to the number of fragments with similar charge/size ratios. McLaughlin et al. [14] published a comprehensive set of practical guidelines for CZE separations in pharmaceutical applications in which they indicated that resolution for tryptic digests of proteins could be significantly improved by the addition of 100 m*M* hexanesulfonic acid to the run buffer. Rush et al. [15] demonstrated similar success using 100 m*M* heptanesulfonic acid for the peptide mapping of recombinant human erythropoietin. Our group has more fully investigated ion pairing in peptide systems by altering the pH of the electrophoretic buffer as well as charge of the ion-pair agent in an effort to ascertain the most important effects governing the enhanced resolution [11]. Fig. 3 shows the separation obtained for a tryptic digest of cytochrome *c* in the presence and absence of 75 m*M* heptanesulfonic acid at pH 2.5. Clearly, resolution is significantly improved in the presence of this anionic ion-pair agent to the extent that the number of resolved components approximately doubles. Under these pH conditions, the majority of

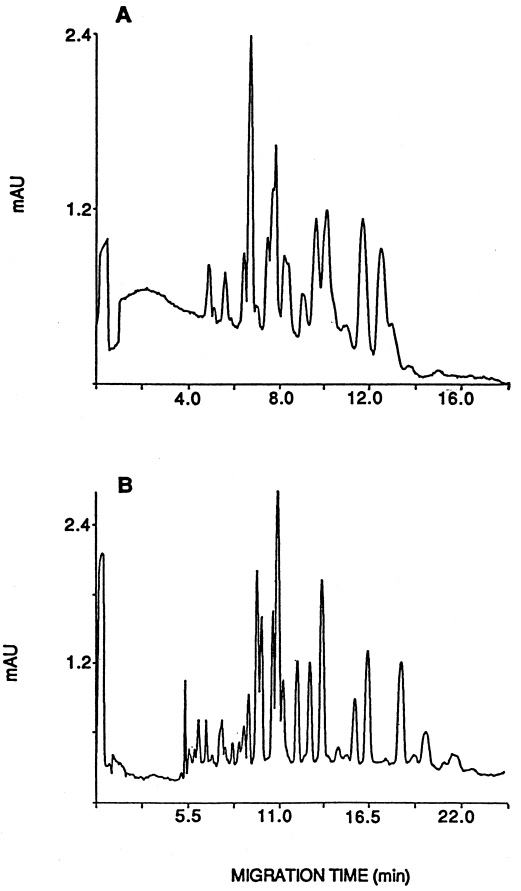

Fig. 3. Separation of a peptide digest of cytochrome *c* in 25 m*M* phosphate, pH 2.5. (A) Buffer only; (B) buffer + 75 m*M* heptanesulfonic acid. (Reprinted from Ref. [11]).

amino acid subunits exhibit a net (+) charge, and thus are amenable to ion-pair formation with the anionic additive. Although some improvement in resolution may be attributed solely to decreased EOF in the presence of the surfactant and reduced wall interaction due to ionic strength effects, it is apparent that selectivity changes are occurring as a result of complexation. Results obtained under basic pH conditions using these same anionic additives demonstrate only marginal improvements in resolution, suggesting that ion–ion interaction (and not hydrophobic association) must play a more dominant role in these separations [11].

As shown in Fig. 1, separations of conventional organic bases such as neurotransmitters can be also improved by the addition of alkylsulfonic acids. We have noted that for small molecule applications, employing surfactants with longer alkyl chains (e.g., decanesulfonic acid) reduces the concentration requirements to achieve similar selectivity effects in a very significant way [11]. Alkylsulfonic acids have also been examined as additives to improve resolution for protein separations with encouraging results. Resolution between cytochrome *c*, albumin and ribonuclease is observed to dramatically improve in pH 2.5 phosphate buffer using uncoated capillaries following the addition of butanesulfonic acid to the run buffer. Separation of the met and oxy forms of human hemoglobin is also facilitated using this additive in a similar buffer matrix. For protein solutes, however, hydrophobic interaction between the alkyl chain of the sulfonic acids and nonpolar amino acid side chains frequently results in denaturation for chain lengths greater than six carbons [11].

Nashabeh and El Rassi [16] investigated the use of the cationic ion-pair agent tetrabutylammonium bromide in the CZE separation of pyridylamino (PA)-derivatized carbohydrate chains of digested α_1-acid glycoprotein. As might be expected, this cation decreases EOF substantially, but also apparently complexes with the derivatized carbohydrate chains through an ion-pair interaction between the sialic acid portion of the carbohydrate and the quartenary ammonium group. This results in vastly improved separations for bovine glycoprotein oligosaccharides as indicated in Fig. 4. Our group has investigated the use of quartenary ammonium salts for separations of phenolic acids and in peptide mapping applications.

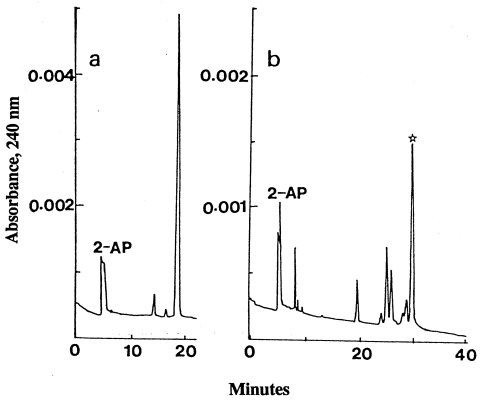

Fig. 4. CZE mapping of pyridylamino derivatives of bovine α_1-acid glycoprotein in 100 m*M* phosphate, pH 5.0. (a) Buffer only; (b) buffer + 50 m*M* tetrabutylammonium bromide. (Adapted from Ref. [16]).

In accordance with Nashabeh and El Rassi, we find that while EOF effects are significant at concentrations of additive above 5 m*M*, selectivity is greatly enhanced for solutes with similar inherent mobilities [11].

For the separation of inorganic ion complexes, Buchberger and Haddad [17] used hexamethonium bromide as a means to simultaneously control EOF and act as an ion-complexing agent for metallo–cyanide complexes and Salimi-Moosavi and Cassidy [18] used tetrabutylammonium hexafluorophosphate (TBAHP) and tetraethylammonium perchlorate (TEAP) to alter selectivity for eleven inorganic cations in non-aqueous solvents. In the latter work, not only was the observed selectivity for these systems dependent on the presence and concentration of the additive, but migration orders actually inverted depending on whether TEAP or TBAHP was used as the complexing agent. These data suggest that ion-pair complexation may be an especially effective means for enhancing separations in pure solvents or solvent combinations less polar than water, where ion–ion interaction is expected to be more extensive.

It is interesting to note that similar additives (i.e., tetraalkylammonium salts) were used in an early application by Wahlbroehl and Jorgenson to effect the separation of neutral organics in acetonitrile–water phases [19]. In this case, separation of five aromatic hydrocarbons was attributed to solvophobic association between the solutes and 25 m*M* tetrahex-

ylammonium ion contained in the run buffer. Since large polyaromatics interact more strongly with the hexyl chains of the cationic additive than the smaller, more polar solutes, they migrate more rapidly through the CZE capillary resulting in complete separation without the need for micelles. Recently, our group has been investigating separations of neutral organics in totally non-aqueous phases including methanol, acetonitrile and formamide, and our data likewise demonstrates the potential utility of hydrophobic interaction. For example, in organic phases containing 100 m*M* tetrahexylammonium bromide, we observe the separation of 3, 4, 5 and 6-membered polyaromatic hydrocarbons in the elution order benzo[*ghi*]perylene, benzo[*a*]pyrene, chrysene and anthracene (Fig. 5) as predicted by Wahlbroehl and Jorgenson [19]. In contrast, when heptanesulfonic acid is used as the additive, the migration order of these compounds is completely inverted as a result of hydrophobic interaction with the complexing anion migrating against EOF. In summary, we reiterate that conventional RP-LC ion-pairing agents can be utilized effectively to improve selectivity for a diversity of CZE applications, although the mode of interaction need not necessarily incorporate ion-pair formation.

3.2. Polymeric ion-pair agents

In their fundamental investigation of ion-pair formation using CZE, Terabe and Isemura [12,13] chose as their ion-pair agents cationic polymers, polybrene (hexadimethrine bromide) and PDDAC (polydiallyldimethylammonium bromide). These polymers, used in concentrations of 0.01–0.06% (PDDAC) or 0.3% (polybrene) are presumed to exhibit their selectivity effects through ion-pair formation with the mono- and dibasic organic acid solutes. Upon addition of the polymers to the aqueous buffer, the direction of EOF is reversed, such that the organic acids are migrating concurrent with EOF, and the complexing agents against it. As shown in Fig. 6, the separation of several organic acids including three naphthalenedisulfonic acids is facilitated in the PDDAC solution. While the authors suggest that the decrease in migration times for the complexed naphthalene derivatives are indicative of greater ion-pairing between these solutes and the cationic polymer versus the other solutes, an alternative possibility suggested by the above discussion is that the nonpolar naphthalene derivatives interact more significantly with the additive as a result of their greater hydrophobic association. It is likely that

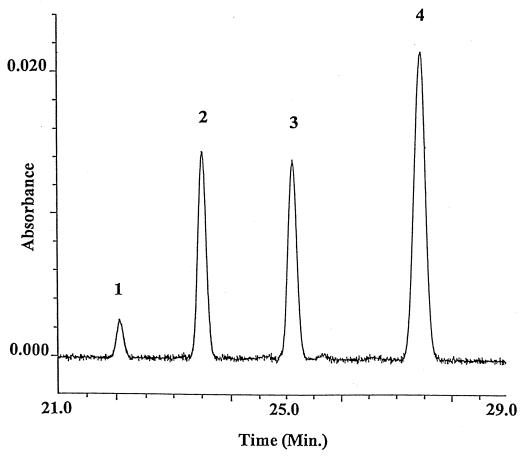

Fig. 5. Separation of (1) benzo[*ghi*]perylene, (2) benzo[*a*]pyrene, (3) chrysene and (4) anthracene in 100% methanol containing 100 m*M* tetrahexylammonium bromide and 25 m*M* sodium acetate. Conditions: 77 cm (70 cm to detection)×50 μm capillary, 30 kV.

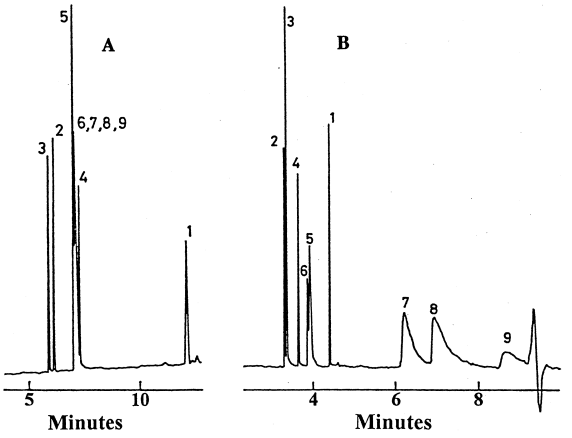

Fig. 6. Separation of dibasic organic acids in 50 m*M* phosphate, pH 7.0, (1) benzoic; (2) maleic; (3) fumaric; (4) phthalic; (5) isophthalic; (6) terephthalic; (7) 2,6-napthalenedisulfonic; (8) 2,7-napthalenedisulfonic; (9) 1,5-napthalenedisulfonic. (A) Buffer only; (B) Buffer + 0.01% PDDAC. (Adapted from Ref. [13]).

both features play some role in increasing the selectivity of the system, although as a practical matter it may be unnecessary to elucidate the relative importance of each mechanism so long as the desired improvement in selectivity is attained.

3.3. Other ion-pair agents

Among other literature citations of the use of ion-pair formation for selectivity enhancement are references to the use of metal ions as complexing additives. As noted in the introduction, a great deal of knowledge of ion-pair interaction for inorganic systems stems from cITP work dating back as early as 1977 [1–7]. In CZE applications, Chen and Pietrzyk [20] and Salimi-Moosavi and Cassidy [21] have used alkali and alkaline earth cations to enhance separations of surfactants in both aqueous and non-aqueous matrices. Both groups have demonstrated that the charge and concentration of the metal ion additives modulate EOF velocity and affect analyte selectivity, resulting in virtually ''tunable'' resolution for surfactant systems. Fig. 7 shows a comparison of a mixture of alkanesulfonates in the presence and absence of Mg^{2+} as a buffer additive. While the reduction of EOF is substantial, migration time is affected to a different degree for each solute, with the short chain surfactants experiencing much greater mobility effects in the presence of the pairing cation [20].

Ion-pair equilibria have also been applied to enhance CZE separations of inorganic cations based on complex formation with small organic acids. Shi and Fritz [22] investigated the use of phthalate, tartrate, lactate and hydroxyisobutyrate as ionic complexing agents for 27 cations, and Fanali and coworkers [4,23] demonstrated the utility of CZE (as well as cITP) for separating isomeric ethylenediamino–amino acid complexes of Co^{3+} using sodium (*S*)-(+)-tartrate as an anionic, chiral complexing agent. We have also investigated the use of chiral dicarboxylic acid salts for inorganic systems, and have recently shown potassium antimonyl *d*-tartrate to be an ideal additive for obtaining chiral resolution among α-diimine transition metal complexes (e.g., Ru^{2+}, Ni^{2+}, Fe^{2+}) [24]. Fig. 8 shows the separation of Λ- and Δ-$Ru(phen)_3^{2+}$ using 100

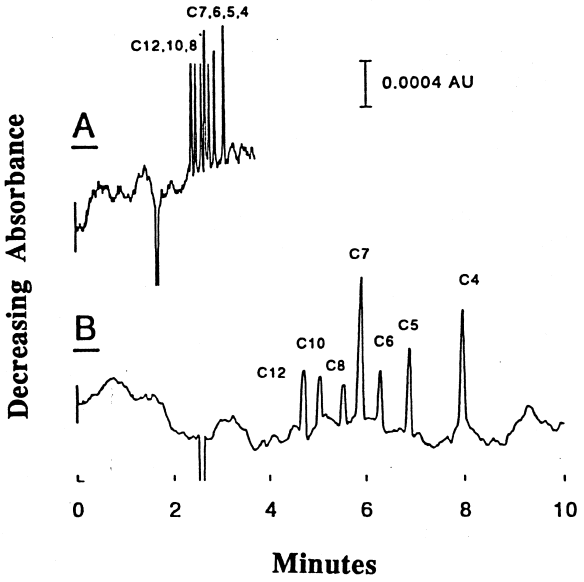

Fig. 7. Separation of C_4–C_{10} alkanesulfonates in 5.0 m*M* phosphate–5.0 m*M* salicylate buffer, pH 7.0 using indirect detection. (A) Buffer only; (B) buffer + 1.00 m*M* Mg^{2+}. (Adapted from Ref. [20] with kind permission from the American Chemical Society).

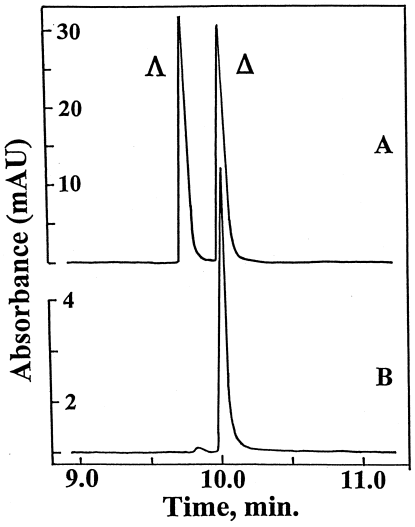

Fig. 8. Electropherograms of (A) racemic $Ru(phen)_3^{2+}$ and (B) Δ-$(-)_D$-$Ru(phen)_3^{2+}$ in 25 m*M* phosphate, pH 7.0 containing 100 m*M* potassium antimonyl *d*-tartrate. (Reprinted from Ref. [24] with kind permission from the American Chemical Society).

m*M* antimonyl tartrate as an anionic complexing additive in the electrophoretic run buffer. Using such an approach, we have demonstrated the capacity of CZE to obtain isomeric resolution for several different metals with similar (or different) ligands in a single injection. Although in each case the stereospecific separation that is obtained is ultimately the result of chiral recognition, it seems likely that it is the attraction of the cationic metal to the anionic ligand (ion-pairing) that initiates the interaction [24].

4. Conclusions

Numerous approaches have been taken to improve CZE separations for ionic solutes with similar inherent electrophoretic mobilities. One of the most effective and easily implemented approaches is the use of ion-pairing agents. Such agents are typically incorporated as salts or soluble polymers in the electrophoretic run buffer, and include conventional RP-LC ion-pair agents. In addition to providing a means of effectively controlling EOF velocity without the need for coated capillaries, solute selectivity may be manipulated based on the appropriate selection of a suitable agent and run buffer concentration. Although numerous manuscripts have been published over the last decade demonstrating specific benefits of such agents, the versatility of the types and applications of ion-pair agents are still being realized. For example, two reports have appeared in this journal as of the preparation of this manuscript in which the authors were able facilitate resolution for complex systems using ion-pair agents in entirely new ways. Greve et al. [25] demonstrated the use of phytic acid to facilitate the separation of charged and neutral underivatized oligosaccharides, and Zhou et al. [26] used lactate as an ion-pairing agent in the chiral separation of a growth hormone secretagogue. It is likely that as increasing numbers of practitioners are familiarized with the diversity and ease of operation of CZE, the need for and interest in improving selectivity will be more acute, and we may anticipate more published accounts of ion-pair enhancement.

Acknowledgements

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