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INDIRECT FLUORIMETRIC DETECTION AND QUANTIFICATION IN CAPILLARY ZONE ELECTROPHORESIS OF INORGANIC ANIONS AND NUCLEOTIDES

LARRY GROSS and EDWARD S. YEUNG*
Department of Chemistry and Ames Laboratory, Iowa State University, Ames, IA 50011 (U.S.A.)

SUMMARY

The combination of indirect fluorescence detection and separation by capillary zone electrophoresis is discussed. Differences in instrument configuration, compared with earlier experiments, resulted in a reversed peak elution order. Separation of a 5′-triphosphate nucleotide from its corresponding diphosphate and monophosphate homologues can be accomplished in 5 min by this method. A quantitative comparison is made between the injection methods of electromigration and injection by gravity flow for samples of inorganic anions diluted in water with varying total concentrations. With injection by electromigration, the amount of sample injected varies significantly with the total ionic concentration of the sample.

INTRODUCTION

An urgent need for the advancement of capillary zone electrophoresis (CZE) is a detection method with wide applicability. Indirect fluorescence detection has been demonstrated to fill this need in certain instances[1,2]. The advantage of this technique is the potential to apply it to solutes that have few other properties that render them observable after being separated[3]. Typical limits of detection (LOD) are in the 50-amol range, which compares favorably with most other detection methods. For example, UV absorption in 50-μm tubes provides at best an LOD of 10 fmol.

One class of solutes that are difficult to detect is simple inorganic anions. Common anions such as chloride, nitrate, phosphate and others can be separated by CZE. They can be detected by measuring refractive index[4], indirect absorption[5] and conductivity[6–8], for example. However, none of these methods works well for very small capillaries or for very low concentrations.

Mikkers *et al.*[9] separated several inorganic anions in a 0.2-mm I.D. capillary in 10 min and detected them using a conductivity detector. In particular, the profile of the peak they measured for Cl^- verified their theoretical derivation of the concentration profile of a solute zone as it elutes through a capillary column[10]. The LOD is only in the picomole range. The extension of their scheme to very small capillaries (< 10 μm) will not be straightforward. The use of smaller capillaries will improve the mass LOD to a level where single-cell studies become feasible. Smaller capillaries also

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favor separation owing to the smaller amount of heat generated during electrophoresis.

Indirect fluorescence detection has been applied to several different liquid chromatographic systems[11,12], because it has the advantages of on-column detection for a minimum dead volume and thus gives more efficient separations, and also because no pre- or post-column derivatization is needed. Displacement of a continuously eluted ionic fluorophore is the mechanism by which solute ions register a signal at the detector[2]. Neutral molecules can also be detected, but with much lower sensitivity owing to the different manner in which uncharged molecules affect the fluorescence signal of the probe. The sensitivity to ions matches well with CZE, as all the separated species are ionic.

In contrast to earlier work, a change in the polarity of the CZE high-voltage potential gradient was needed in order to separate small, fast-moving anions. This had the additional benefit of increasing the usefulness of the technique for separations of nucleotides, which were also reported previously[2,13]. As a consequence, it is now possible to separate mono-, di- and triphosphate nucleotides in a single CZE run. We also report here the quantitative nature of indirect detection, including the dynamic range and various injection bias effects[14].

EXPERIMENTAL

The CZE instrument used has been described previously[1,2]. About 10 mW from an ultraviolet argon ion laser (Model 2035 Spectra-Physics, Mountain View, CA, U.S.A.) is now used to excite on-column fluorescence. A prism is used to separate its output in the UV range at 331 nm from two stronger lines at around 350 nm. A knife edge selects the 331-nm beam, which then enters a laser power stabilizer (Cambridge Research and Instrumentation, Cambridge, MA, U.S.A.). The stabilized beam is focused on the capillary by a 1-cm focal length lens and the fluorescence is collected using a 20 × microscope objective lens. The configuration of these optics differs from that in the reference cited[2] in that a five-fold improvement in the dynamic reserve was obtained by eliminating an aperture for spatial filtering of light scattered from the walls of the capillary. This aperture was mounted between the objective lens and the photomultiplier tube (PMT) (R928, Hamamatsu, Middlesex, NJ, U.S.A.). The fluorescence emission turned out to be sufficiently different in wavelength from the excitation scattered light that two long-pass filters (Schott Glass, Melles Griot, Rochester, NY, U.S.A.) removed at least 99% of the scattered light.

A second difference from the CZE methodology previously used[1,2] is the direction in which ions are eluted in the column. A succinct rationale for the change in elution order had recently appeared[15]. Previously, injections were made at the anode (which was the high-voltage electrode) and electroendosmosis (EEO) carried anions to the cathode. The electropherograms in this work were obtained by injecting at the cathode and detecting at the ground-potential anode. The linear velocity of the migrating anions was greater than the velocity of EEO in the opposite direction. A lower pH buffer (3–4) was used and it was helpful for reducing the EEO flow-rate[16] compared with the previous work[2].

The buffer was prepared by mixing in an equal molar ratio solutions of salicylic acid and sodium salicylate, obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.) and

Fisher Chemical Co. (Fair Lawn, NJ, U.S.A.), respectively. The pH varied from 4.0 for 0.25 m*M* salicylate buffer (SAL) to 3.5 for 1.0 m*M* SAL. Fused-silica capillaries were purchased from PolyMicro Technologies (Phoenix, AZ, U.S.A.).

RESULTS AND DISCUSSION

Detection of inorganic anions and nucleotides

The electropherogram in Fig. 1 shows the separation of seven anions with concentrations ranging from 8 to 54 μM in a sample. Oxidizing agents were chosen for the test sample to minimize redox reactions between the sample components. The elution times for the peaks in the test mixture are given in Table I, together with the concentrations. The inside wall of the capillary used for this separation was untreated.

In order to obtain the separation shown in Fig. 1, it was necessary to reduce the injection time to 0.7 s from the 1 s usually used for injection by electromigration. The peak width and hence the efficiency were governed by the duration of injection. The efficiency was calculated to be N (plate number) = 25 000 from the peak width at the baseline for the phosphate peak.

The speed of the separation depended on whether or not the column was silylated[2]. The velocity of EEO was reduced when columns were treated according to procedures described elsewhere[17]. Because the ions are migrating "upstream" relative

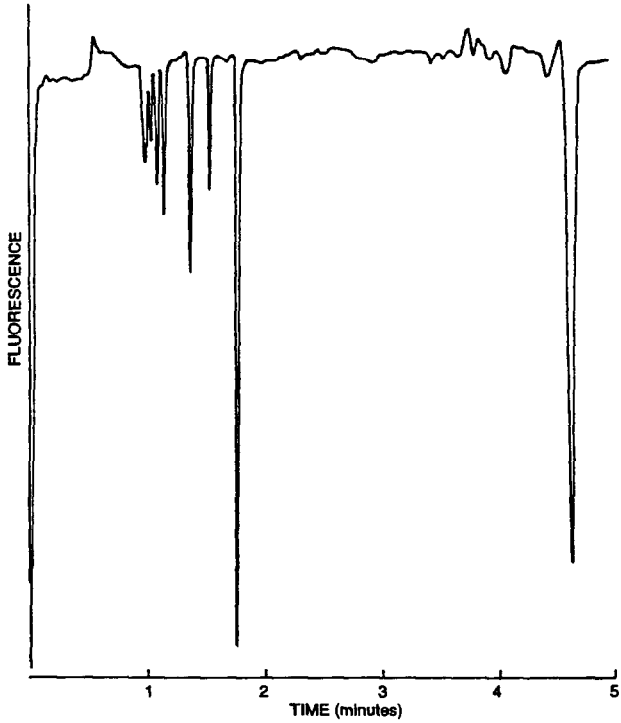

Fig. 1. Separation of inorganic anions by CZE. Conditions are listed in Table I.

TABLE I

ELUTION ORDER FOR INORGANIC ANIONS

Conditions: operating buffer, 0.25 m*M* equimolar mixture of salicylic acid and sodium salicylate (pH 4.0); column, overall length 51.2 cm × 14 μm I.D.; injection (cathode) end to detector, 33.1 cm; injection, 0.7 s at 30 kV; electrophoresis, 5.7 nA at 30 kV; dynamic reserve, 1300.

Anion	*Elution time (s)*	*Concentration (μM)*
Cl^-	53.0	19.0
NO_3^-	54.6	9.0
ClO_4^-	56.0	25.0
MnO_4^-	64.6	12.0
$Cr_2O_7^{2-}$	76.4	8.0
IO_3^-	88.6	12.0
$H_2PO_4^-$	102.6	54.0
Salicylate	270.0	–

to the EEO flow, as a result the elution times for anions were decreased when columns were treated. The system peak also eluted faster when the column was silylated.

The elution time of the system peak corresponds to the effective mobility of the salicylate ions in the column, in addition to the flow-rate due to EEO. Note that salicylate, like the other anions, migrates towards the anode. Increasing the proportion of sodium salicylate to salicylic acid in the buffer solution increased the pH slightly. The result was a larger fraction of salicylate ions and therefore an increased effective mobility of these ions. The system peak was observed to elute more quickly when water was injected with the buffer, which is a direct result of an increase in pH.

The system peak was largest when water alone was injected. When the sample concentration was increased, again for samples diluted in water, the system peak decreased in area. This peak even reversed and became positive-going if the sample concentration was sufficiently large, that is, of the order of that of the running buffer. At low analyte concentrations, the injection diluted the buffer and reduced the fluorescence of salicylate. At high sample concentrations, the extra cations "sweep along" more salicylate ions than were normally present in the buffer, reversing the sign of the system peak.

With the buffer mixture used here, the system peak was always the last peak to be eluted after an injection, whether by electromigration or by gravity injection. This agrees with the relative mobilities of all the ions. When hydrodynamic injection (gravity or siphon flow injection) was used, the size of the system peak was increased relative to the areas obtains for the sample peaks. This was expected as dilution is more important for hydrodynamic injection than for electromigration.

The buffer concentration affects the elution time of the peaks through the slow velocity of EEO in two ways. A lower buffer concentration gives a slightly higher buffer pH (owing to dissolved carbon dioxide and hence hydrogencarbonate ions), and increasing pH is known to increase the EEO flow-rate (owing to high charge densities at the capillary walls)[16]. Also, the EEO flow-rate is inversely proportional to ionic concentration, so decreasing the buffer concentration gives an increase in EEO flow-rate[18]. Accordingly, the elution times were observed to increase when the SAL concentration was reduced to 0.125 m*M*.

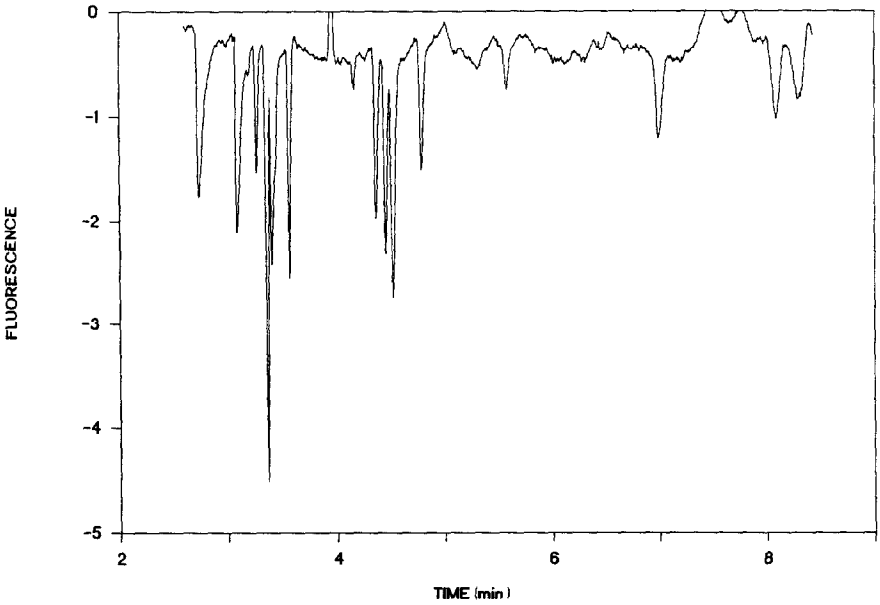

Fig. 2. Separation of twelve nucleotides by CZE. Conditions are listed in Table II.

The control of the EEO flow-rate via the buffer concentration was implemented to obtain the electropherogram in Fig. 2. The separation of a sample of twelve mono- and diphosphate nucleotides is shown. The peak elution times are listed in Table II. For this separation, the buffer concentration was increased to 1.0 m*M* in order to reduce the EEO flow-rate by approximately half compared with the 0.25 m*M* SAL used for the first electropherogram. The EEO flow-rate was slow enough that all four mono-, di- and triphosphate nucleotides could be eluted under the same conditions. In Table II, the retention times are listed for each of the nucleotides separated in Fig. 2. The peaks were identified by injecting samples of the mono-, di- and triphosphate series of each base in combination and alone.

TABLE II

RETENTION TIMES (t_R) FOR NUCLEOTIDE SEPARATION SHOWN IN FIG. 2

Conditions: operating buffer, 1.0 m*M* SAL (pH 3.5); column, overall length 68.1 cm × 14 μm I.D., silylated; injection end (cathode) to detector, 46.3 cm; injection, by electromigration for 0.6 s at 30 kV.

Sample	t_R *(s)*	*Sample*	t_R *(s)*
Thymidine 5′-monophosphate (TMP)	268	Adenosine 5′-diphosphate (ADP)	263
Guanosine 5′-monophosphate (GMP)	288	Cytidene 5′-diphosphate (CDP)	272
Adenosine 5′-monophosphate (AMP)	421	Uridene 5′-triphosphate (UTP)	165
Cytidene 5′-monophosphate (CMP)	485	Guanosine 5′-triphosphate (GTP)	186
Thymidene 5′-diphosphate (TDP)	196	Cytidene 5′-triphosphate (CTP)	202
Guanosine 5′-diphosphate (GDP)	214	Adenosine 5′-triphosphate (ATP)	205

Calibration graphs for phosphate

Standard solutions were prepared from sodium dihydrogenphosphate. Peak areas were measured by triangulation of the peaks from the recorder chart. Seven standard solutions were injected by gravity flow for 5 min. The running buffer was 0.25 m*M* salicylate. For concentrations ranging from 1 to 120 m*M* the calibration graph was linear with slope = 0.12 $cm^2/\mu M$ passed through the origin. The correlation coefficient was $r^2 = 0.994$. This shows that indirect fluorescence is quantitative and can be used for concentrations that are only half of the total buffer concentration, or equal to that of the ionized species.

To calculate the LOD for this series of injections, Poiseuille's law was used to calculate the volume of solution injected. This assumption was checked by observing the progress of a sample of riboflavin in a capillary of I.D. 25 mm under gravity flow. The velocity of the flow was found to agree with that predicted by Poiseuille's law to within 30%. With similar accuracy the zone injected into a 14-μm column was calculated to be 5 mm in length and to contain 600 amole of $H_2PO_4^-$ when the concentration was 0.9 μM. The LOD was 0.2 μM of $H_2PO_4^-$ injected or approximately 130 amol injected.

The standard solutions discussed above were prepared by diluting dihydrogenphosphate ion in water. The calibration graph obtained by injecting these solutions by electromigration was not linear, except at low phosphate concentrations of 1–10 μM. When the standard solutions were diluted in the buffer (0.25 m*M* SAL), the calibration graph for injection by electromigration was linear but the sensitivity was markedly reduced. The difference is shown in Fig. 3, where peak areas are plotted against phosphate concentration for samples diluted in water, in buffer and in 1.0 m*M* Cl^- solution, for both electromigration injection (A) and gravity injection (B). In Fig. 3A, the larger peak areas imply that more phosphate was injected on the column when the sample was diluted in water, The intermediate peak areas were obtained when phosphate was dissolved in the running buffer. This is consistent with the fact that electric field strength (which determines the migration velocity at injection) decreases on going from water to buffer to 1.0 m*M* chloride.

In Fig. 3B, the overall difference in size of the peaks obtained was smaller than in Fig. 3A. Also, the intermediate peaks are those for phosphate dissolved in 1.0 m*M* Cl^-. This differs from Fig. 3A, where the intermediate response is from phosphate dissolved in buffer. A possible explanation for this difference is the effect of pH of the sample compared with the pH of the running buffer. For the running buffer and the standards dissolved in SAL buffer, the pH was calculated to be about 4, based on the acid dissociation constant of salicylic acid at this concentration. The pH of the phosphate sample dissolved in water or in 1.0 m*M* Cl^- is approximately 5, because it is controlled by the equilibrium of dissolved carbon dioxide. Presumably, the solutions with higher pH contained a larger fraction of phosphate anions, with the result that more salicylate anions were displaced. Because the signal in indirect fluorescence detection is based on charge displacement on injection, Fig. 3B reflects the differences in ionic equilibrium for the phosphate ion in different pH environments.

Effect of other ions present

In injection by electromigration, the concentration of the sample solution has been shown[14] to affect the peak areas measured for components separated by CZE

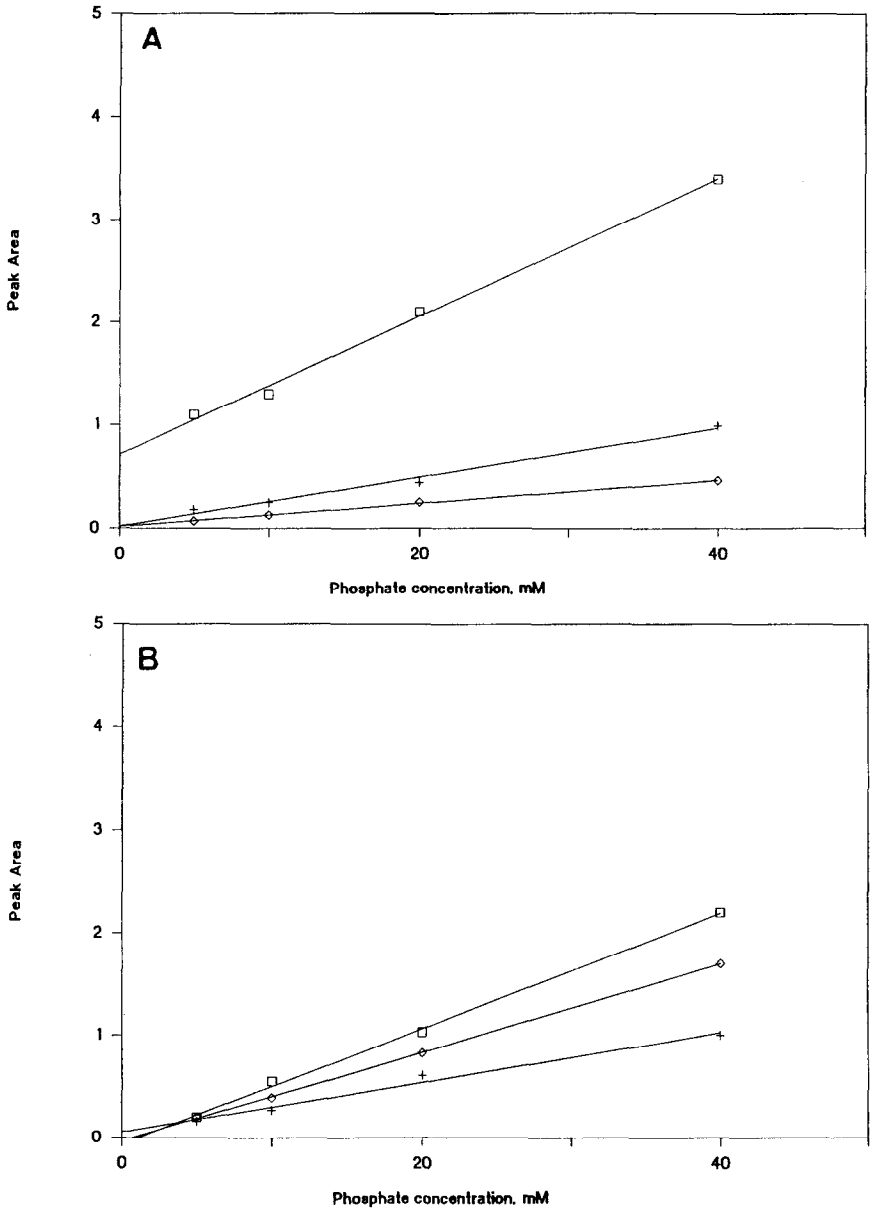

Fig. 3. Comparison of linearity and intercepts of calibration graphs for $H_2PO_4^-$ dissolved in (□) water, (◇) 1.0 m*M* chloride and (+) buffer (250 μM) for (A) electromigration and (B) gravity injection.

and detected by conductivity. The lower the total concentration of the injected sample, the higher was the sensitivity that was obtained. This effect was related to the conductivity of the sample solution, although the EEO flow-rate was also possibly involved there. The EEO flow-rate in the present experiments was in the opposite direction to the motion of the anions, however, and should not be a factor here.

A series of solutions were prepared in which the concentration of $H_2PO_4^-$ was kept fixed at 20 μM. The Cl^- concentration for these solutions was varied from 20 μM to 1.0 mM. The samples were diluted with water and injected by electromigration for 1 s at 30 kV. The peak areas for the phosphate peak decreased rapidly with increasing Cl^- concentration. A second series of samples were prepared in the oppo-

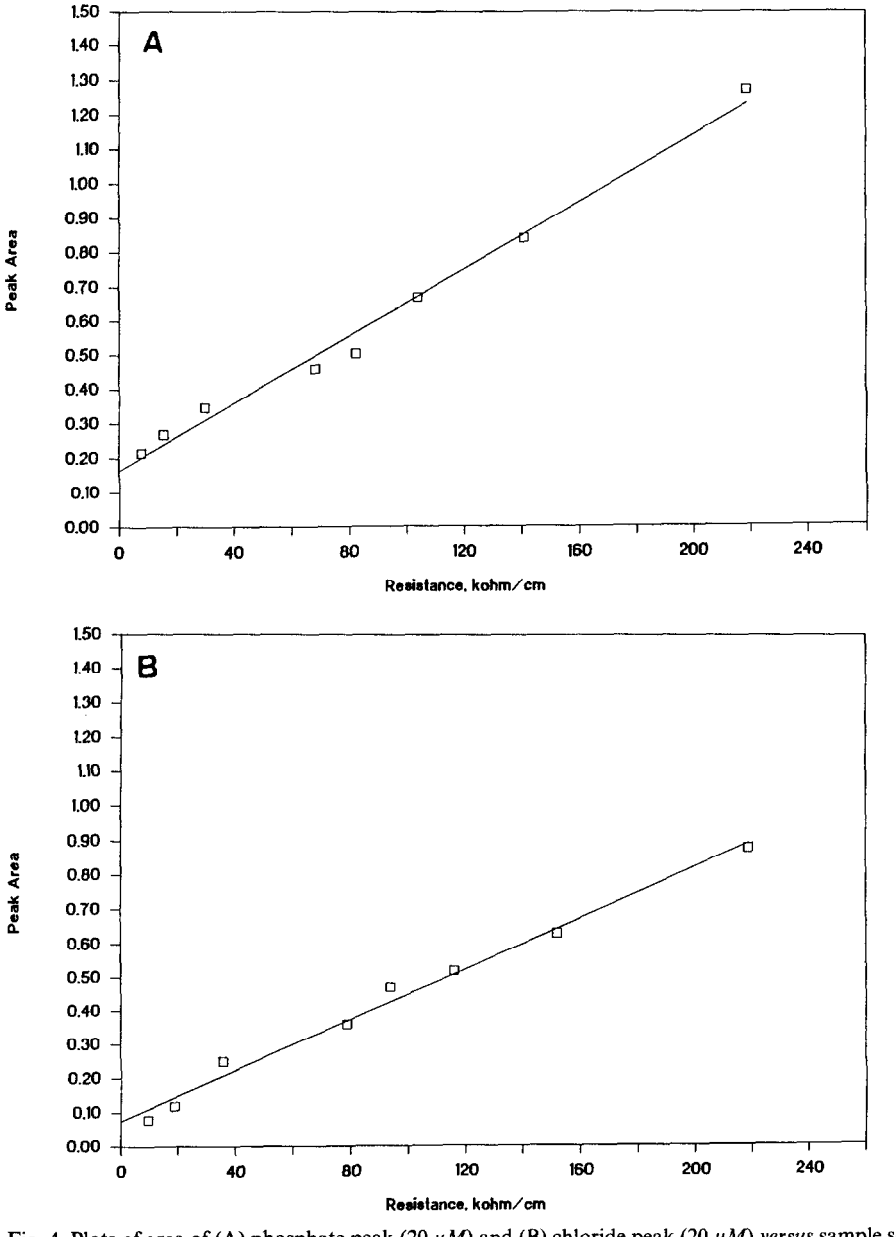

Fig. 4. Plots of area of (A) phosphate peak (20 μM) and (B) chloride peak (20 μM) *versus* sample solution resistance.

site sense, with the Cl^- concentration held fixed at 20 μM and the concentration of an equimolar mixture of $H_2PO_4^-$ and HPO_4^{2-} increased from 20 μM to 1.0 mM (total phosphate concentration).

Correlations were then made of the peak areas of the phosphate and chloride peaks against the resistance of the sample solution. The resistance of each solution was calculated by adding up the ionic conductivities of each of the positive and negative ions in the sample, multiplied by their concentration. When the measured peak area was plotted against the resistance, straight lines were obtained. These plots are shown in Fig. 4A and B for the area of the phosphate and chloride peak, respectively. The results are consistent with higher field strengths at higher solution resistance, causing more ions to be injected.

CONCLUSIONS

The separation of anions can be accomplished using a polarity for the CZE instrument opposite to that described in previous work. By using conditions that decrease the EEO flow-rate, anions can be eluted in a direction opposite to the EEO flow. The EEO flow-rate was reduced in this instance by reducing the buffer pH. Column treatments have been developed that are also effective in reducing EEO flow-rates at higher pH[17].

The most significant aspect of the separations demonstrated here is the sensitivity for anions at such low concentrations and in such small molar amounts. Because a smaller column is used, the absolute amount detected is much smaller than that by alternative methods, such as refractive index or conductivity. Also, as the number of ions present in the focus of the laser is such a small fraction of the number injected, the potential exists for much higher sensitivity, provided that narrower zones can be injected and baseline stability can be improved. The 10^{-16} mol or 10^{-7} M LOD achieved here should make CZE competitive with ion chromatography in many applications.

The dependence of analyte peak areas and the concentration of other ions in the sample is a drawback when electromigration is used for injection. The separation in Fig. 1 was not achieved when gravity flow injection (5 min) was attempted. This is due to the parabolic profile for hydrodynamic flow. The trade-off between quantification of well separated peaks with gravity flow injection and the need for narrow injection plugs that are easily obtained with electromigration injection is therefore an important consideration.

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