ELSEVIER

Journal of Chromatography B, 657 (1994) 409–417

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of metal ions by capillary electrophoresis using on-column complexation with 4-(2-pyridylazo)resorcinol following trace enrichment by peak stacking

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Abstract

Co^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+} were separated and detected following derivatization with 4-(2-pyridylazo)resorcinol by capillary electrophoresis with UV–Vis detection. Parameters which were examined include both on-column and pre-column complexation, limit of detection, capillary loadability, linear dynamic range and reproducibility. A sample stacking technique was investigated in order to obtain better detection limits and greater sensitivity. Detection limits of $1 \cdot 10^{-8}$ *M* were achieved for Co^{2+}, Fe^{2+} and Zn^{2+} and $4 \cdot 10^{-7}$ *M* for Cu^{2+}. Mass detection limits were 0.2 fmol for Co^{2+}, Fe^{2+} and Zn^{2+} and 7.0 fmol for Cu^{2+} The use of this method for the determination of metals in vitamin tablets and a pond water sample is presented.

1. Introduction

Currently, the most utilized technique for separation and determination of metal ions as their chelates is reversed-phase liquid chromatography (RPLC) with spectrophotometric detection [1]. Several chelating agents have been employed as derivatization reagents in metal ion analysis, including dithiocarbamic acids, 8-hydroxyquinoline and β-diketones [1]. 4-(2-Pyridylazo)resorcinol (PAR) has been employed as a chelating agent with both precolumn [2–4] and postcolumn [5–7] derivatizations. PAR forms water-soluble complexes with more metal ions than does any other commonly available indicator [3]. It is a tridentate ligand with the donor atoms being the pyridine nitrogen, the azo nitrogen furthest from the pyridine ring, and the ortho-hydroxyl oxygen.

In recent years, capillary electrophoresis (CE) has become a recognized and useful tool in the area of biological analysis [8]. However, metal ion analysis using CE has not been widely reported. The determination of metal cations has been accomplished using CE with indirect detection [9,10]. Although this method allows sensitive detection of cations, it is not very selective. A number of metal ions have been determined directly by CE with electrochemical detection (ED) using a mercury microelectrode [11]. Although detection limits were in the micromolar range for most metals, problems with long-term stability of the electrodes restrict the usefulness of this method.

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0378-4347/94/$07.00
SSDI 0378-4347(94)00098-T

A drawback of direct detection methods is that electrostatic interactions of the uncomplexed metal ions with ionized silanol groups can lead to band broadening or loss of analyte. In order to minimize this interaction, investigators have added the chelator α-hydroxyisobutyric acid to the run buffer [9,11]. An alternative approach for the determination of metals by CE is to use a chromophoric chelating reagent. This eliminates the problem of electrostatic interactions as well as "derivatizing" the metal for spectrophotometric detection. Two papers have reported the use of chelating agents in capillary zone electrophoretic separations of metal ions. 8-Hydroxyquinoline-5-sulfonic acid [12] was employed with laser-induced fluorescence detection for the determination of Ca^{2+}, Mg^{2+} and Zn^{2+} with limits of detection in the micromolar range. The determination of Ni^{2+} and Co^{3+} using PAR and CE–UV has also been reported [13]. Cu^{2+}, V^{5+} and Fe^{2+} were also included in this study; however, the Cu^{2+} peak was not resolved from the reagent peak and no quantitation data on the other metals were provided. Limits of detection for Ni^{2+} and Co^{3+} were $1.4 \cdot 10^{-7}$ and $1.8 \cdot 10^{-7}$ *M*, respectively. It was mentioned in this paper that PAR complexes of Cd^{2+}, Mn^{2+} and Zn^{2+} were unstable and could only be detected if PAR were added to the run buffer, but no electropherogram was presented.

In 1989, Zelenský *et al.* [14] employed on-column complexation with xylenol orange for the photometric detection of Zn^{2+}, Mn^{2+} and Cd^{2+} during capillary isotachophoresis. More recently, we employed on-column complexation with Cu^{2+} for the selective detection of peptides by capillary zone electrophoresis with ED [15]. In addition, it has been shown by others that peak stacking is a very effective method for trace enrichment in CE [16,17]. In this paper both methods are used for the detection and separation of several metal ions (Co^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+}) by CE. On-column complexation with PAR combined with peak stacking provides a 10- to 100-fold reduction in limits of detection over precolumn complexation methods. In order to demonstrate the applicability of the method, the procedure was applied to the analysis of a vitamin tablet and a pond water sample.

2. Experimental

2.1. Apparatus

Fused-silica capillaries, 50 μm I.D. × 300 μm O.D., were obtained from Polymicro Technologies (Phoenix, AZ, USA). Prior to use, these capillaries were treated with 0.1 *M* NaOH. The high-voltage power supply was a Glassman MJ30P400 power supply (Glassman High Voltage, Whitehouse Station, NJ, USA), the input from which was placed in a Plexiglas box with an interlock in the access door for safety. UV–visible on-column detection with a C4 capillary electrophoresis absorbance detector (ISCO, Lincoln, NE, USA) was carried out at 500 nm. The total length of the capillary was 85 cm and the length to the detector was 75 cm.

2.2. Reagents

The stock metal ion solutions were prepared by dissolving metal salts of nitrates (Fe^{2+} and Co^{2+}), sulfate (Cu^{2+}) or acetate (Zn^{2+}) (Aldrich, Milwaukee, WI, USA) in Nanopure water (Sybron-Barnstead, Boston, MA, USA). An electrophoretic buffer of 10 m*M* N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS) (Sigma, St. Louis, MO, USA) was used throughout the investigation; the pH was adjusted using NaOH. The working PAR solution was prepared by dissolving the reagent in electrophoretic buffer at pH 8.4.

2.3. Procedure

Precolumn complexation

For precolumn formation of PAR metal chelates, an appropriate quantity of metal ion stock solution was added to 10 m*M* TAPS buffer, pH 8.4, containing $1 \cdot 10^{-3}$ *M* PAR. A 10-fold excess of reagent was used to ensure complete complexation. Chelates of all metal ions formed immediately at room temperature, and were injected within 30 min of preparation. A lower concentration of PAR ($1 \cdot 10^{-4}$ *M*) was added to the electrophoretic buffer to prevent the dissociation of relatively unstable chelates such as those of Zn^{2+} and Cu^{2+}. The applied voltage

was 30 kV, resulting in a current of 5 μA. Solutions were injected onto the capillary electrokinetically at 10 kV for 10 s.

On-column complexation

On-column complexation involved a three-step sequence. In the first step, a 1 m*M* solution of the ligand was injected onto the capillary for a period of 10 s at 10 kV. The sample solution containing the metal ions was then injected electrokinetically for 15 s at 10 kV. Lastly, the anodic end was once again placed in the run buffer (containing 0.1 m*M* PAR) and 10 kV was applied for a 5-s period (pause time). This ensured adequate complexation of the ligand and the metal ion. Subsequently, the voltage was ramped to 30 kV to effect the separation. For sample stacking, samples were injected in Nanopure water, employing the three-step sequence previously described, and complexation occurred on column.

2.4. Sample preparation

Commercial vitamin supplements in tablet form (Prenatal formula with zinc; Nature Made Nutritional Products, Los Angeles, CA, USA) were dissolved in water and filtered using a 0.2-μm filter. Subsequently, a volume of the sample filtrate was added to the PAR solution; this solution was then injected electrokinetically onto the capillary.

The water samples were obtained from a small pond in Lawrence, KS, USA. They were spiked with $1 \cdot 10^{-7}$ *M* Fe^{2+} and Zn^{2+} and acidified with HCl to release any trace of metal ions bound to organic matter. They were then injected directly into the CE system.

3. Results and discussion

3.1. The effect of pH on complex stability

PAR complexes exhibit greatest stability at alkaline pH. Under acidic conditions (pH < 5), the PAR reagent degrades completely, thus inhibiting complexation. The pH range 8–10 provides the greatest chelate stability; however, resolution of the different metal chelates is compromised at very high pH due to the high electroosmotic flow. Therefore, a pH of 8.4 was chosen for the separations. This provided adequate resolution and good ligand stability.

3.2. Electrophoretic buffer composition

Phosphate, borate and TAPS were investigated for separation of the metal complexes. Higher separation currents and lower chromatographic efficiencies were evident with the use of phosphate and borate buffers. In contrast, the zwitterionic buffer TAPS exhibited much smaller separation currents (5 μA) and resulted in more efficient separations.

Because some of the metal–PAR chelates *e.g.*, Zn^{2+} and Cu^{2+}, were unstable [4,13], a low concentration of PAR ($1 \cdot 10^{-4}$ *M*) was added to the electrophoretic buffer to enhance the separation. Table 1 illustrates that higher efficiencies are obtained when PAR is used in the run buffer, than when it is absent. As a result of this increase in efficiency, the metal ions were much better resolved with the inclusion of PAR in the electrolyte. The presence of the reagent in the run buffer at this concentration did not result in higher background absorbance, and therefore did not reduce detection sensitivity.

3.3. Metal ion separations

Fig. 1 illustrates the separation of Co^{2+}, Cu^{2+}, Fe^{2+} and Zn^{2+} and free PAR following precomplexation, with electrokinetic injection of the

Table 1
Effect of inclusion of PAR in the electrophoretic buffer on separation efficiency and resolution

Metal	PAR present		PAR absent	
	N	R_s	*N*	R_s
Co^{2+}	58 564	6.0	27 777	1.9
Cu^{2+}	34 348	1.2	1 038	0.3
Fe^{2+}	99 575	1.16	14 208	0.4
Zn^{2+}	106 711	1.16	3 696	0.4

N = Number of theoretical plates; R_s = Resolution between neighboring peaks.

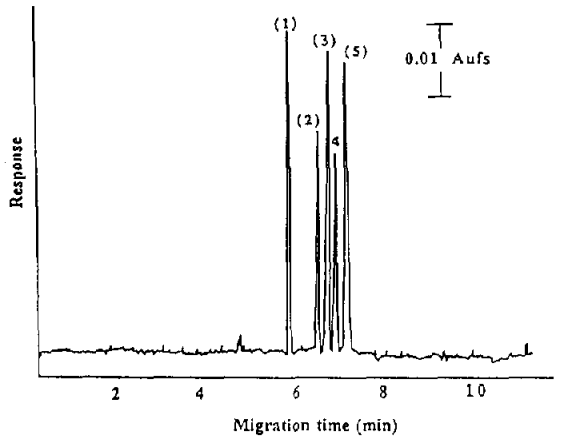

Fig. 1. Separation of metal–PAR chelates with precomplexation. Peaks: 1 = Co^{2+}; 2 = free PAR; 3 = Cu^{2+}; 4 = Fe^{2+}; 5 = Zn^{2+}. Electrophoretic buffer, 10 m*M* TAPS and $1 \cdot 10^{-4}$ *M* PAR, pH 8.4. Sample pH, 8.4; applied voltage, 30 kV; metal concentration, $4 \cdot 10^{-5}$ *M*; [PAR] $1 \cdot 10^{3}$ *M*; Electrokinetic injection at 10 kV, 10 s.

chelate. This separation is an improvement over that reported by Iki *et al.* [13] in that Cu^{2+} is resolved from the PAR reagent. The calculated efficiencies are lower, but this could be due to the lower field strength employed in our separation. Calibration curves of all metal ion chelates showed linear dynamic ranges from $1 \cdot 10^{-6}$ to $1 \cdot 10^{-4}$ *M*, with an average correlation coefficient of 0.9996. The detection limits ($S/N = 2$) were $1 \cdot 10^{-6}$ *M* for Zn^{2+}, Fe^{2+} and Co^{2+} and $3 \cdot 10^{-6}$ *M* for Cu^{2+}. Six injections of a $4 \cdot 10^{-5}$ *M* solution provided an average R.S.D. of 1.8%.

The poor limits of detection obtainable with this method are partly attributable to the small pathlengths characteristic of capillary columns. Incorporation of a z-cell or bubble cell could increase the effective pathlength and enhance the detection sensitivity. An additional factor contributing to the poor limits of detection reported here is the poor quality of the lamp in this detector. For these studies a deuterium lamp was employed which has little power output at 500 nm. A lamp or laser providing more power at 500 nm would greatly increase the sensitivity of the method. The average wavelength selected for detection of the metal ions was 500 nm since this was a wavelength common to all the metal species. If the wavelength maxima specific for each individual species were employed, *e.g.*, Co^{2+} (500 nm), Cu^{2+} (506 nm), Fe^{2+} (540 nm) and Zn^{2+} (490 nm), the sensitivity for that particular analyte could be improved.

3.4. Sample analysis

The applicability of the precomplexation procedure to real samples was confirmed through the analysis of a commercial vitamin tablet (Fig. 2). The correlation between expected and observed results was very good for the six samples analyzed. The values obtained were 286 mg/l for Fe^{2+} and 118 mg/l for Zn^{2+}. These were only slightly lower than the expected values based on the product label, which were 300 mg/l and 125 mg/l, respectively. The R.S.D. for six vitamin samples was 2.0 and 2.6% for Fe^{2+} and Zn^{2+}, respectively.

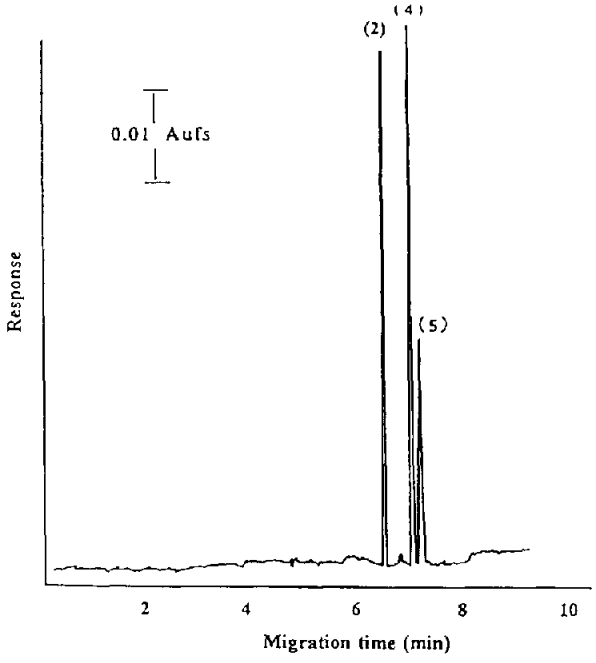

Fig. 2. Electropherogram of metal ion separation in a commercial vitamin supplement. Precomplexation with PAR. [PAR] $1 \cdot 10^{-3}$ *M*. Electrophoretic buffer, 10 m*M* TAPS with $1 \cdot 10^{4}$ *M* PAR, pH 8.4. Peaks: 2 = free PAR; 4 = Fe^{2+}; 5 = Zn^{2+}.

3.5. On-column complexation with peak stacking

To obtain adequate limits of detection for water analysis (pg/ml), most LC-based methods for the determination of metal ions involve a trace-enrichment step. The most common procedures use bonded-phase or ion-exchange resins [6,7] to concentrate the sample off-line. In CE, peak stacking has been shown to be an effective trace enrichment method. Sample stacking occurs as a result of the movement of sample ions across the stationary boundary, which separates the region of the injected sample solution from the rest of the capillary containing the support buffer [16,18]. Because of the matrix differences between those two regions, the ions experience a lower electric field in the support buffer region than in the sample region, thus the velocity of the ions decreases as they cross the stationary boundary. The slower-moving ions will "stack up" into a smaller volume, thereby increasing the concentration in the sample zone [19].

To obtain the best sensitivity with the PAR reagent, a three-step stacking procedure was developed, which involved on-column complexation of the metal and PAR (Fig. 3). In order to maximize the amount of complexation, it was discovered that an initial plug of higher concentration PAR ($1 \cdot 10^{-3}$ M) was necessary, followed by a plug of metal ions in water. The reason for this is that the positively charged metal ions have a positive electrophoretic mobility, and therefore move more rapidly toward the cathode than does PAR (which has a negative electrophoretic mobility), thus mixing with the slower migrating negatively charged ligand. Without this initial plug of PAR, the buffer containing $1 \cdot 10^{-4}$ M ligand carries the metal ions, but the kinetics are not fast enough for complete complexation to occur. There could also be stacking of metal ions due to the presence of the PAR zone [20].

The effect of the PAR zone is clearly illustrated in Fig. 4a. In this case, no plug of PAR was introduced prior to the metal ion, and peak heights are substantially reduced. Even more importantly, under these conditions the metal ions which form relatively weak chelates, *e.g.*, Cu^{2+} and Zn^{2+} [13], do not have sufficient time to complex, and this results in broad peaks. Fig. 4b shows the satisfactory CE separation of the metal chelates under the stacking conditions, where a 10-s electrokinetic injection of 1 mM PAR is introduced prior to the metal ion. The advantage of introducing a more concentrated plug of PAR is demonstrated by this electropherogram.

On-column complexation, with the metal ions injected in buffer identical to the electrophoretic buffer, achieved detection limits similar to those obtained with precolumn complexation. The effect of sample buffer concentration on peak stacking was investigated, and it was observed that the peak heights increased as the buffer concentration in the sample decreased. In this study, the optimum stacking effect was observed with pure water.

In order to optimize the on-column complexation procedure, the effect of injection time and voltage on peak height and column efficiency for the detection of Co^{2+} was investigated. Injection times were varied between 5 and 35 s at 5-s intervals. As can be seen in Fig. 5, a maximum was reached at 15 s. Above this period, excess metal ion is introduced, resulting in a reduction in peak height. The injection voltage also had an influence on the efficiency of the separation.

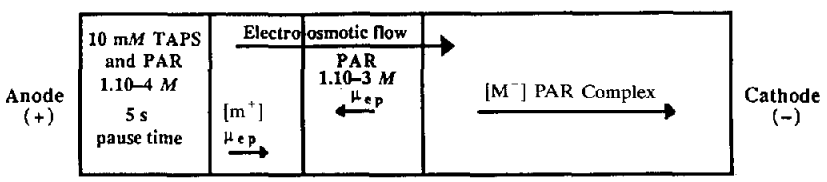

Fig. 3. Schematic diagram of stacking with on-column complexation; μ_{ep} is the electrophoretic mobility.

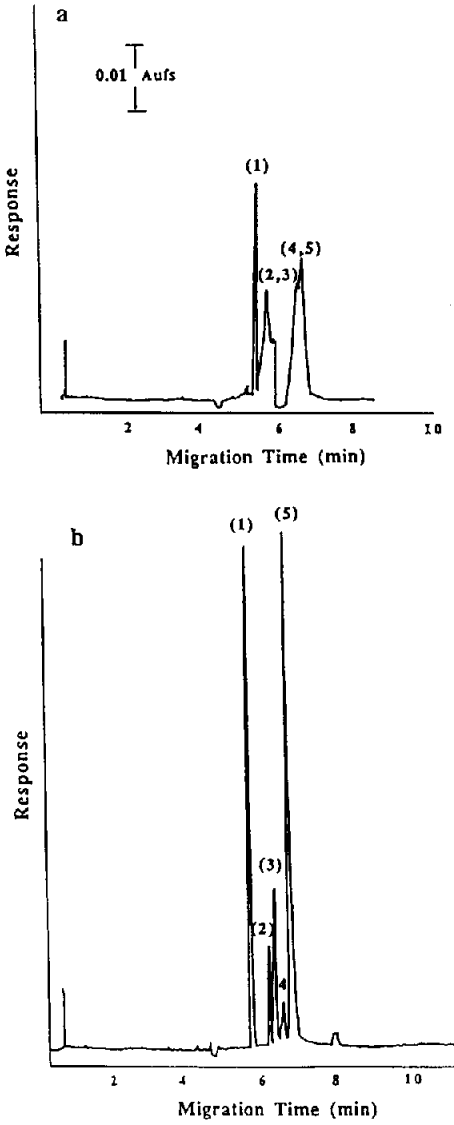

Fig. 4. (a) CE separation under stacking conditions in the absence of PAR plug. (b) CE separation of metal–PAR chelates under stacking conditions and on-column complexation. Peaks: 1 = Co^{2+}; 2 = free PAR; 3 = Cu^{2+}; 4 = Fe^{2+}; 5 = Zn^{2+}. Stacking voltage, 10 kV; injection times, [PAR] $1 \cdot 10^{-3}$ *M*, 10 s; [M]$^{+}$ $8 \cdot 10^{-7}$ *M*, 10 s; [PAR] $1 \cdot 10^{-4}$ *M*.

Obviously, the higher the voltage the greater the quantity of metal introduced, and, therefore, the larger the peak height. However, resolution became a factor at higher injection voltages as the peak width increased substantially with an increase in voltage. At voltages above 10 kV the

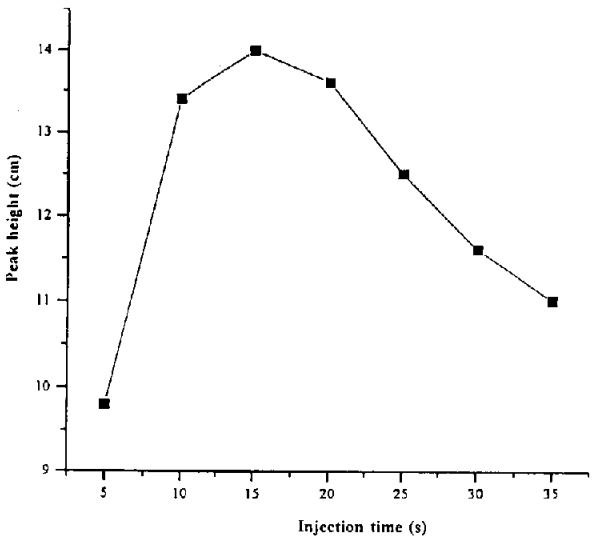

Fig. 5. Optimization of sample injection time during peak stacking. Co^{2+} $1 \cdot 10^{-6}$ *M*; stacking sequence, first plug [PAR] $1 \cdot 10^{-3}$ *M*, 10 s Co^{2+}; pause time, 5 s. Electrophoretic buffer 10 m*M* TAPS containing $1 \cdot 10^{-4}$ *M* PAR. Stacking voltage, 10 kV.

efficiency was dramatically reduced (see Fig. 6). At 5 kV a sufficient quantity of ions was not introduced onto the capillary. Therefore, based

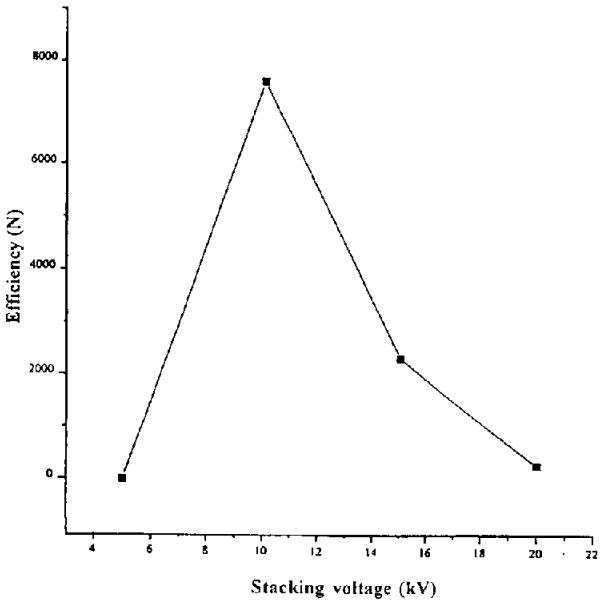

Fig. 6. Effect of injection voltage on separation efficiency under stacking conditions. *N* = Number of theoretical plates.

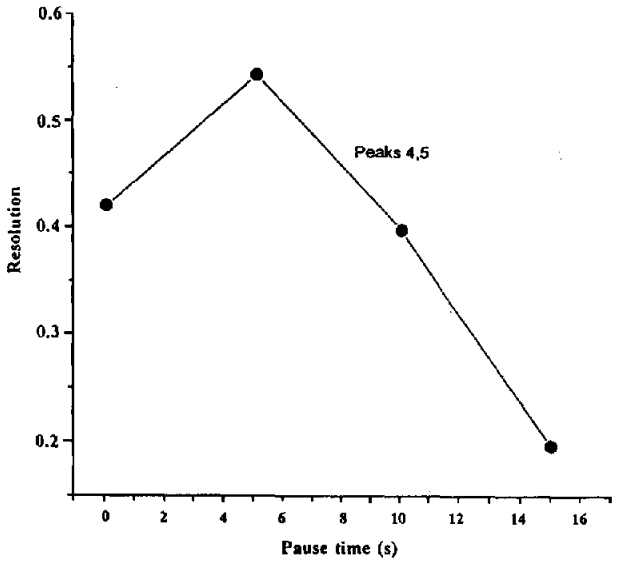

Fig. 7. The effect of pause time on resolution under peak stacking conditions; 5 s. Electrophoretic buffer, 10 m*M* TAPS containing $1 \cdot 10^{-4}$ *M* PAR, pH 8.4.

on these experiments, a 10-kV electrokinetic injection for 15 s was determined to best provide both satisfactory baseline resolution and peak height response.

The third step in the stacking program involved a pause time, whereby the run buffer is applied at 10 kV for a period of 5 s, after which the voltage was ramped to 30 kV to allow separation to continue. If this step is omitted, complexation is incomplete. On the other hand, too large a pause interval results in a dramatic reduction in both resolution and efficiency. Fig. 7 shows the effect of pause time on the resolution of Fe^{2+} and Zn^{2+}. A pause time of 5 s was determined to be optimal.

3.6. Limits of detection

The combination of peak stacking and on-column complexation resulted in a 100-fold reduction in detection limits for Co^{2+}, Fe^{2+} and Zn^{2+} and a 10-fold reduction for Cu^{2+}. Detection limits were $1 \cdot 10^{-8}$ *M* for Co^{2+}, Fe^{2+} and Zn^{2+} and $4 \cdot 10^{-7}$ *M* for Cu^{2+} ($S/N = 2$). These correspond to mass detection limits of 0.2 fmol for Co^{2+}, Fe^{2+} and Zn^{2+} and 7.0 fmol for Cu^{2+}. Reproducibility of peak heights ranged from 0.8 to 1.9% R.S.D. for the metal ions studied. The linear dynamic range for these metals was greater than three orders of magnitude with an average correlation coefficient of 0.9987 (Table 2). Even though there was a substantial loss of efficiency when the stacking method was employed, as can be seen in Fig. 4b, the peaks are still adequately resolved for quantitation.

3.7. Water samples

The use of this method for the analysis of a pond water sample was investigated. No Fe^{2+} or Zn^{2+} was found to be present in the original sample, so it was spiked with $1 \cdot 10^{-7}$ *M* metal

Table 2
The effect of peak stacking on limit of detection (LOD) ($S/N = 2$)

Metal	Precomplexed[a]		Stacking	
	LOD (*M*)	R.S.D. (%)[b]	LOD (*M*)	R.S.D. (%)[b]
Co^{2+}	$3 \cdot 10^{-6}$	0.9	$1 \cdot 10^{-8}$	1.0
Cu^{2+}	$1 \cdot 10^{-6}$	3.1	$4 \cdot 10^{-7}$	0.8
Fe^{2+}	$1 \cdot 10^{-6}$	2.5	$1 \cdot 10^{-8}$	1.9
Zn^{2+}	$1 \cdot 10^{-6}$	1.0	$1 \cdot 10^{-8}$	1.0
Linear range	$1 \cdot 10^{-6}$–$1 \cdot 10^{-4}$ *M*		$1 \cdot 10^{-8}$–$1 \cdot 10^{-5}$ *M*	
Average correlation coefficient	0.9996		0.9987	

[a] Without stacking.
[b] Concentration of metal ion $4 \cdot 10^{-5}$ *M*.

ion. Fig. 8a shows the electropherogram obtained using precolumn complexation. Neither ion was detectable. Fig. 8b shows the same sample using on-column complexation and peak stacking. In this case Fe^{2+} and Zn^{2+} are both discernible. There was some reduction in the sensitivity of the method when using the real sample. This is believed to be due to the addition of the acid to release trace metal ions which could be bound to organic matter.

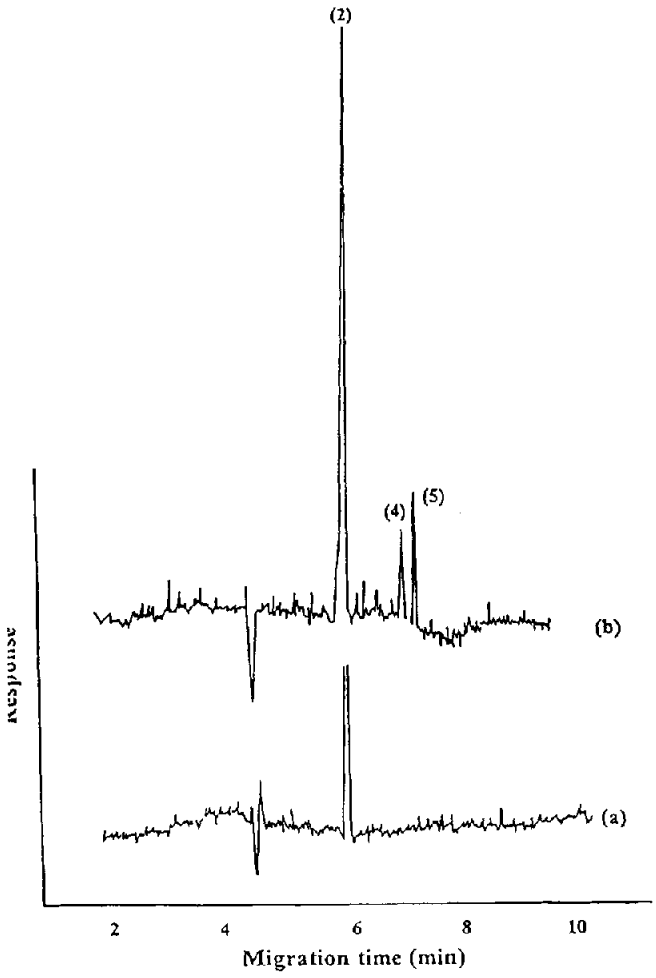

Fig. 8. Water sample spiked with $1 \cdot 10^{-7}$ *M* Fe^{2+} and Zn^{2+} and analyzed by (a) precolumn complexation and (b) on-column complexation and peak stacking. Conditions as in Fig. 5.

4. Conclusions

The technique described provides a rapid, simple and sensitive technique for determination of trace metal ions in aqueous samples. Using sample stacking and on-column complexation, it is possible to achieve a 10-fold reduction in detection limit for Zn^{2+} and 100-fold reductions for Cu^{2+} and Co^{2+}, compared to precolumn complexation. The detection limits are an order of magnitude lower than those reported using the same chelating reagent [13] and better than those reported using indirect detection [9,10] or electrochemistry [11]. In addition, the chelator PAR exhibits greater selectivity than that which can be obtained with indirect detection. The applicability of this technique to real samples was confirmed through the analysis of vitamin supplements and a pond water sample.

5. Acknowledgements

This work was funded in part by the Kansas Technology Enterprise Corporation. The authors thank Paul Weber, Tommy O'Shea and Doug Monroe for their technical assistance. We would also like to thank Nancy Harmony for her help in the preparation of this manuscript.

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