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# Isotachophoretically Assisted On-Line Complexation of Trace Metal Ions in a Highly Saline Matrix for Capillary Electrophoresis†

**Jihye Kim, Kihwan Choi, Sunyoung Cho, Asif Riaz, and Doo Soo Chung***

*Department of Chemistry, Seoul National University, Seoul 151-747, Korea. *E-mail: dschung@snu.ac.kr*
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Trace metal ions such as $Cd^{2+}$, $Ni^{2+}$, and $Zn^{2+}$ in a highly saline sample were subjected to on-line complexation with 4-(2-thiazolylazo) resorcinol (TAR) dissolved in a background electrolyte (BGE) under transient isotachophoresis (TITP) conditions. A long plug of the saline sample, containing the trace metal ions but devoid of TAR, was injected into a coated capillary filled with a BGE composed of 150 mM 2-(cyclohexylamino) ethanesulfonic acid (CHES) and 110 mM triethylamine (TEA) at pH 9.7. Since the electrophoretic mobility of TAR fell between the mobilities of the anionic leading electrolyte ($Cl^-$ in the sample) and the anionic terminating background electrolyte ($CHES^-$), a highly concentrated zone of TAR from the BGE was formed at the rear of the sample matrix and then the metal cations toward the cathode were swept by isotachophoretically assisted on-line complexation (IAOC) between the metal ions and the isotachophoretically stacked TAR. As a result, anionic metal-TAR complexes were formed efficiently, which satisfy the TITP conditions between $Cl^-$ and $CHES^-$. The enrichment factors of metal ions including $Cd^{2+}$ were up to 780-fold compared to a conventional CZE mode using absorbance detection. The detection limits were 17 nM, 15 nM, and 27 nM for $Ni^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ in a 250 mM NaCl matrix, respectively. Our method was successfully applied to the analysis of urine samples without desalting.

**Key Words :** Transient isotachophoresis, Salt, Trace metals, Cadmium, 4-(2-Thiazolylazo) resorcinol

## Introduction

For the determination of trace metal ions in capillary electrophoresis (CE), chromophoric complexation has typically been used since most metal ions are neither naturally UV/Vis absorbing nor fluorescent.[1,2] Previously, we have reported transient isotachophoresis (TITP) of trace metal ions such as $Ni^{2+}$, $Fe^{2+}$, and $Zn^{2+}$ complexed with 4-(2-pyridylazo)resorcinol (PAR) in a high salt matrix.[3,4] Metal-PAR complexes were prepared off-line and subjected to TITP stacking, utilizing the sample matrix chloride as the leading electrolyte (LE) to achieve limits of detection (LOD) in the low/sub ppb range. However, the method was not applicable to some heavy metal ions such as $Cd^{2+}$ due to its weak complexation with PAR.[5,6] 4-(2-Thiazolylazo)resorcinol (TAR), having a thiazolylazo moiety instead of the pyridylazo moiety, shows strong complexation with metal ions such as $Cd^{2+}$, $Zn^{2+}$, and $Ni^{2+}$ and has been used for CE of those metal ions.[6-8] When the trace metal ions were enriched first by field enhanced sample injection and then on-line complexed with preloaded TAR, LOD's were reduced to the low/sub ppb range.[8] This scheme required that samples should be dissolved in a dilute matrix and thus entailed difficulty for use with physiological samples which are usually saline.

In this report, we demonstrate isotachophoretically assisted on-line complexation (IAOC) for the determination of trace metals including $Cd^{2+}$ in a highly saline matrix. A long plug of a highly saline sample devoid of the complexing agent TAR was directly injected to obtain a 330-fold enrichment for Ni-TAR, 780-fold for Zn-TAR, and 420-fold for Cd-TAR achieving LODs in the low nanomolar range. Furthermore, this IAOC method was applied to the analysis of real biological samples such as human urine without desalting.

## Experimental

**Reagents.** 2-(Cyclohexylamino)ethanesulfonic acid (CHES) and triethylamine (TEA) were purchased from Sigma (St. Louis, MO, USA). Highly pure sodium chloride (99.999%), nickel(II) chloride hexahydrate, zinc nitrate hexahydrate, cadmium nitrate tetrahydrate, and TAR were from Aldrich (Milwaukee, WI, USA). 2,7-Dichloro-fluorescein (DCF) was from Merck (Darmstadt, Germany). Fluorinated carbon polymer neutral (FC-PN) which is a 50 g/L aqueous solution of FC-430 (3M, St. Paul, MN, USA) was purchased from J&W Scientific (Folsom, CA, USA). All reagents were used as received. A reference material for trace metals in urine (ME 28351) was obtained from Promochem (Wesel, Germany).

The background electrolyte (BGE) for CE was a pH 9.7 buffer composed of 150 mM CHES and 110 mM TEA containing 0.02 vol % FC-PN and 0.2 mM TAR. A 1 mM stock solution of TAR was prepared in deionized water with a few drops of 1 M NaOH (subject to complete dissolution)

Abbreviations: CHES: 2-(Cyclohexylamino)ethanesulfonic acid, DCF: 2,7-Dichlorofluorescein, PAR: 4-(2-Pyridylazo)resorcinol, FC-PN: Fluorinated carbon polymer neutral, TAR: 4-(2-Thiazolylazo) resorcinol, TEA: Triethylamine, TITP: Transient isotachophoresis, IAOC; Isotachophoretically assisted on-line complexation

†This paper is to commemorate Professor Kook Joe Shin's honourable retirement.

before volume make-up. Appropriate amounts of TAR stock solution and FC-PN had been added before adjusting the pH to 9.7 with TEA. 7 mM stock solutions of $Ni^{2+}$, $Zn^{2+}$, and $Cd^{2+}$ were prepared in deionized water. The sample solution for IAOC was prepared in a 0.6-mL plastic vial by adding 50 $\mu$L of 1 M NaCl and the analyte stock solutions, and then the total volume was made up to 200 $\mu$L with water, followed by mixing with a vortex mixer. A standard solution of 100 $\mu$M metal-TAR was prepared in BGE by diluting the stock solutions. Morning urine from a healthy volunteer was collected in a plastic bottle. 10 $\mu$L of urine was mixed with the 190 $\mu$L of a standard matrix solution containing 250 mM NaCl and appropriate amounts of spiked analytes. All solutions were filtered through a 0.45-$\mu$m syringe filter (Whatman, Clifton, NJ, USA) and degassed by sonication prior to CE runs.

**Capillary Electrophoresis.** CE analyses were carried out with a P/ACE 5500 system (Beckman, Fullerton, CA, USA). All CE experiments were performed under negligible EOF conditions. A $\mu$-Sil-FC coated fused silica capillary (50 $\mu$m ID, 365 $\mu$m OD) from Agilent (Waldbronn, Germany) was also provided with FC-PN as an additive to the BGE for dynamic coating during electrophoresis, serving as a protective shield for the permanent coating. The polyimide coating at 50 cm from the injection end of a 57 cm capillary was removed with hot sulfuric acid to create a detection window.

A new $\mu$-Sil-FC coated capillary was filled with 0.05 vol % FC-PN in water and left for 5 h for capillary conditioning. Before the first run of the day, the capillary was rinsed with 0.02 vol % FC-PN for 5 min at $1.38 \times 10^5$ Pa. Before sample injection, the capillary was rinsed with BGE for 3 min at $1.38 \times 10^5$ Pa. Then, a sample solution was introduced hydrodynamically at $3 \times 10^3$ Pa. Separation was carried out at a constant current mode in reverse polarity (−30 $\mu$A). The capillary was thermostated at 20°C during electrophoresis. Absorbance at 500 nm was monitored for the detection of metal-TAR peaks. Since the large sample volume might have changed the BGE composition in the inlet and outlet vials, the BGE was replenished and a fresh sample was used for each run. The capillary was rinsed with 0.02 vol % FC-PN for 5 min at $1.38 \times 10^5$ Pa after each run.

The electrophoretic mobilities of free-TAR, Ni-TAR, Zn-TAR, Cd-TAR, $Cl^-$, and $CHES^-$ were measured at a constant voltage mode applying +25 kV across the capillary. For the indirect absorbance mode to monitor $Cl^-$ and $CHES^-$ at 254 nm, 5 mM chromate was added to the BGE. Free-TAR, Ni-TAR, Zn-TAR, and Cd-TAR were monitored at 500 nm. DMSO was used as an EOF marker.

## Results and Discussion

**IAOC of Metal Ions in a Highly Saline Matrix.** Doubly deprotonated $TAR^{2-}$ acts as a tridentate ligand forming complexes with most transition metals, predominantly in a 2:1 ratio of TAR to a metal ion[9,10]

$$M^{2+} + 2TAR^{2-} \rightleftharpoons [M(TAR)_2]^{2-}. \quad (1)$$

Figure 1 shows the structure of a metal-TAR complex and the absorption spectra of TAR and metal-TAR complexes such as Ni-TAR, Zn-TAR, and Cd-TAR measured in a 150 mM CHES/110 mM TEA buffer (pH 9.7). This chromophoric complexation reaction has been used to determine trace metal ions in dilute aqueous samples. By on-line complexation of a plug of concentrated TAR and metal ions stacked at the boundary between the sample and TAR plugs, the LOD's were reduced to the low/sub ppb range for $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$, and $Fe^{2+}$ dissolved in deionized water.[8] However, for dense samples, such as a commercial zinc gluconate solution used as a dietary supplement, the sample had to be diluted 5000-fold before injection. Here we report a scheme for sensitive determination of trace metal ions including $Cd^{2+}$ in a highly saline matrix without complicated pretreatment steps such as desalting. A long plug of a trace metal sample in 250 mM NaCl is swept by IAOC between the metal ions and TAR using $Cl^-$ in the sample as an LE, yielding LODs in the low nanomolar range.

In isotachophoresis (ITP), a sample solution is injected between a LE with the highest mobility and a terminating electrolyte (TE) with the lowest mobility:

$$|\mu_L| > |\mu_S| > |\mu_T|, \quad (2)$$

where $\mu$ is the electrophoretic mobility of the species denoted by the subscript L, S, and T for LE, sample, and TE, respectively. TITP is a technique in which solutes are initially concentrated by ITP and then separated by capillary zone electrophoresis (CZE) in a single capillary.[11-14] In other words, a heterogeneous system of electrolytes satisfying Eq. (2) exists transiently, followed by the restoration of a homogeneous BGE system for CZE. One scheme of TITP involves a BGE ion serving as a LE or TE, while a sample ion serves as TE or LE, respectively. Samples of natural

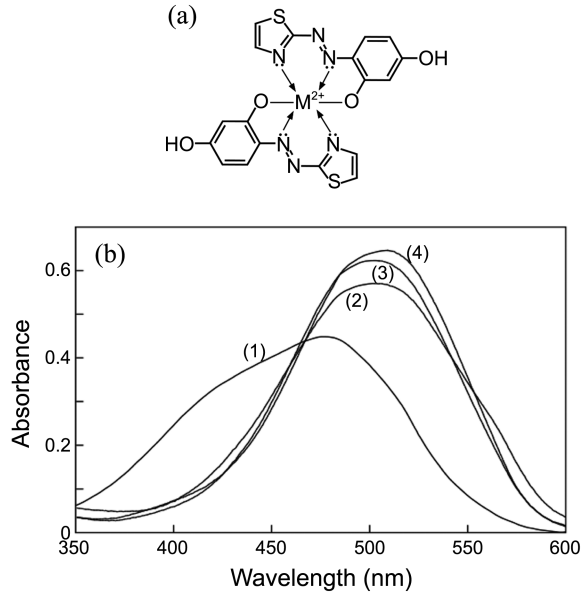


**Figure 1.** (a) Structure of a metal-TAR complex. (b) Absorbance spectra of 16 $\mu$M (1) TAR, (2) Ni-TAR, (3) Zn-TAR, and (4) Cd-TAR in 150 mM CHES, 110 mM TEA, and 0.2 mM TAR buffer of pH 9.7.

origin such as body fluids often contain highly mobile $Cl^-$ at sufficient concentrations for use as a LE in an appropriate system of BGE. Various applications of TITP to highly saline biological samples have been reported including the analysis of trace metal complexes.[3,4,15-18]

The experimentally measured electrophoretic mobilities of anionic species were $\mu_{Cl} = -65.3 \times 10^{-9}$ m$^2$/Vs, $\mu_{TAR} = -38.3 \times 10^{-9}$ m$^2$/Vs, $\mu_{Ni\text{-}TAR} = -29.2 \times 10^{-9}$ m$^2$/Vs, $\mu_{Zn\text{-}TAR} = -29.0 \times 10^{-9}$ m$^2$/Vs, $\mu_{Cd\text{-}TAR} = -28.7 \times 10^{-9}$ m$^2$/Vs, and $\mu_{CHES} = -21.7 \times 10^{-9}$ m$^2$/Vs. The order of the electrophoretic mobilities is

$$|\mu_{Cl}| > |\mu_{TAR}| > |\mu_{M\text{-}TAR}| > |\mu_{CHES}|. \quad (3)$$

Based on the above relation, we propose the mechanism of IAOC depicted in Figure 2. A long plug of a saline sample, but devoid of TAR, is injected into a capillary filled with the BGE. After placing the inlet and outlet of the capillary into vials containing the BGE, a reverse potential is applied. Then, transient isotachophoresis is initiated using the chloride in the sample as an LE and CHES as a TE. As the leader chloride moves towards the outlet, a plug of isotachophoretically enriched TAR follows behind. The height of the free-TAR peak showed a strong dependence on the chloride concentration in the sample, with a linear correlation coefficient of 0.992 in the concentration range of 100 to 400 mM (data not shown). Metal cations, which are moving towards the inlet, encounter the TAR plug and are converted into anionic species by on-line complexation with TAR as Eq. (1). The isotachophoretically enriched TAR assures the trace level of metal ions are swept clearly. Then, according to Eq. (3), narrow isotachophoretic plugs of metal-TAR will follow the TAR plug in order of the mobilities. As the transient isotachophoretic condition is broken, CZE proceeds in the same capillary. Note that, in this IOAC scheme, the TAR concentration in the BGE can be kept low and thus the baseline noise can be reduced, yielding high *S*/*N* ratios for the metal-TAR peaks.

The BGE composition was optimized by varying the CHES concentration from 50 to 200 mM with an interval of 50 mM.

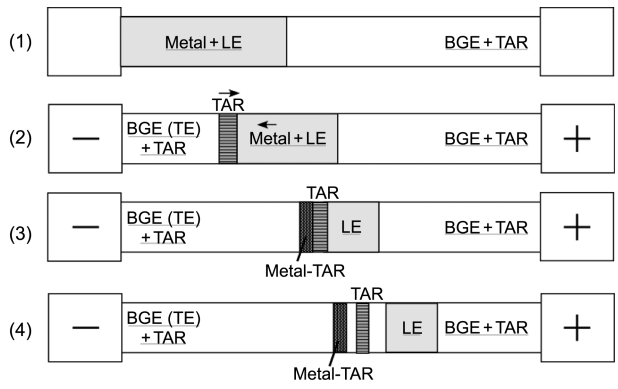


**Figure 2.** IAOC of metal ions in a highly saline matrix. (1) Injection of a sample plug containing chloride as an LE. (2) Free-TAR is stacked between the LE and TE at the rear of the sample plug. (3) IAOC of metal and TAR (4) Concentrated metal-TAR and free-TAR are separated in the CZE mode.

At a lower concentration of CHES a fronting behavior of the TAR peak was observed. However, as the concentration of CHES was increased, the fronting of the TAR peak diminished; peak efficiencies and resolutions between the metal-TAR peaks were improved. To avoid excessive Joule heating 150 mM of CHES was chosen as optimal. The BGE pH affected peak efficiencies to a considerable extent. At a lower pH, the peak efficiencies of Ni-TAR and Zn-TAR were good, whereas Cd-TAR showed poor peak efficiency; however, the opposite was true at a higher pH. Thus the pH of the BGE was kept at 9.7 for all the experiments.

Electropherograms of Ni-TAR, Zn-TAR, and Cd-TAR complexes obtained in the CZE and IAOC modes are compared in Figure 3. CZE was conducted by injecting a 100 $\mu$M sample dissolved in BGE for 1 s at $3 \times 10^3$ Pa, while the IAOC scheme was performed with a plug of 1 $\mu$M metal ions in a 250 mM NaCl, but without TAR, injected for 420 s at $3 \times 10^3$ Pa. Despite the fact that the metal-TAR complexes are stable, a substantial dissociation could be seen during electrophoretic migration (data not shown). Therefore, the BGE for CE was supplemented with 0.2 mM TAR and the peak shapes were significantly improved. A higher concentration of TAR in BGE was avoided due to excessive baseline noise. Metal-TAR complexes were efficiently formed under highly saline conditions and the detection sensitivities were increased 330-fold for Ni-TAR, 780-fold for Zn-TAR, and 420-fold for Cd-TAR. The efficiencies of the complex peaks were typically in the range 220 000-520 000 under IAOC, while they were in the range 170 000-310 000 under CZE. The efficiencies of the peaks in IAOC were about 1.5 times higher, even with a sample plug 420 times longer than the one in the CZE mode.

**Analytical Performance.** The linear dynamic ranges with

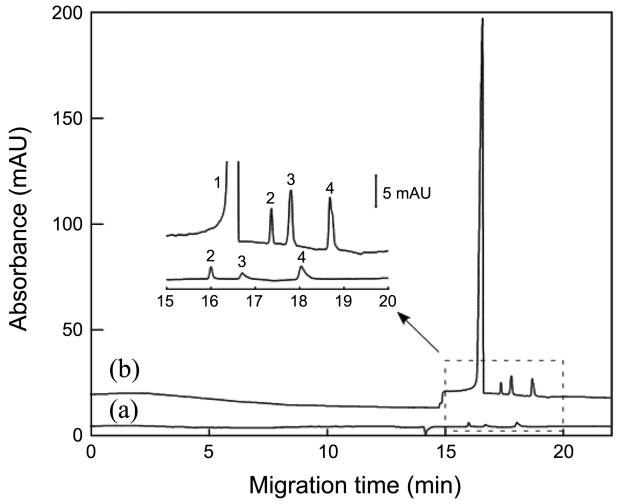


**Figure 3.** Electropherograms for (a) the CZE mode injecting 100 $\mu$M samples in BGE for 1 s at $3 \times 10^3$ Pa and (b) the IAOC mode injecting a large volume of 1 $\mu$M trace metal in 250 mM NaCl for 420 s at $3 \times 10^3$ Pa. Peaks: 1; Free TAR, 2; Ni-TAR, 3; Zn-TAR, and 4; Cd-TAR. BGE: 150 mM CHES, 110 mM TEA, 0.2 mM TAR, and 0.02 vol % FC-PN (pH 9.7). $\mu$-Sil-FC coated capillary: 50/57 cm, 50 $\mu$m ID, thermostated at 20°C. Detection: 500 nm. Separation current: –30 $\mu$A.

the peak areas were 17-14000 nM for Ni-TAR, 15-15000 nM for Zn-TAR, and 44-8900 nM for Cd-TAR. The linear correlation coefficients were in the range of 0.997-0.998 for an injection of 420 s at $3 \times 10^3$ Pa. Each complex has a low nM level LOD ($S/N = 3$); 17 nM for Ni-TAR, 15 nM for Zn-TAR, and 27 nM for Cd-TAR. The quantification results are listed in Table 1.

For the validation of the proposed method, a reference material for trace metals in urine (ME 28351) was analyzed by the standard addition method. In order to ensure the TITP condition for a urine sample of undetermined salt concentration, the sample was diluted with solution containing 250 mM NaCl. Due to the high concentration of Zn in ME 28351, the reference urine was analyzed with serial dilution up to 20 times. The obtained concentration values were close to the assigned composition of ME 28351 and within the recommended confidence ranges of the double standard deviation established by independent laboratories of clinical chemistry (see Table 2). The RSDs of the migration times were 0.1-0.2%. The reproducibility in quantification of a reference sample in a day was 0.6-7.5% RSD ($n = 3$) and the day-to-day reproducibility was 2.1-9.7% RSD ($n = 3$).

**Human Urine Analysis.** Human urine samples were analyzed using the IAOC method. In general, biological samples should be desalted prior to the injecting step in a conventional CE analysis. However, a high level of chloride in the sample can be advantageous if it acts as a LE for IAOC. Figure 4 shows TITP stacking of metal-TAR complexes in a urine sample. The 1:20 diluted urine sample contained DCF as an internal standard. Since the chloride concentration in urine varies over time and depends on eating and drinking habits, the urine sample was diluted with the solution containing 250 mM NaCl. Furthermore, the additional amount of NaCl in the sample ensures the onset of TITP which should be at not less than a critical level.[11,17] Electropherograms in Figure 4 show the analysis results of spiked human urine samples by our IAOC method. Figure 4a was obtained from a urine sample diluted 20-fold with 250 mM NaCl containing 500 nM DCF, $Ni^{2+}$, and $Cd^{2+}$ but devoid of $Zn^{2+}$. Thus the Zn-TAR peak was from urinary zinc. Figures 4b to 4e show peaks identified by additional spiking with 500 nM DCF, $Ni^{2+}$, $Zn^{2+}$, and $Cd^{2+}$, respectively. In multiple analyses of one urine sample, the migration time reproducibility was 0.1% RSD ($n = 4$). Urine samples collected on different days showed slightly lower reproducibility in migration times (RSD 0.1-0.6%, $n = 3$). The RSD values for intraday and interday quantification of urine samples were 2.8-10.0% ($n = 4$) and 2.2-11% ($n = 3$).

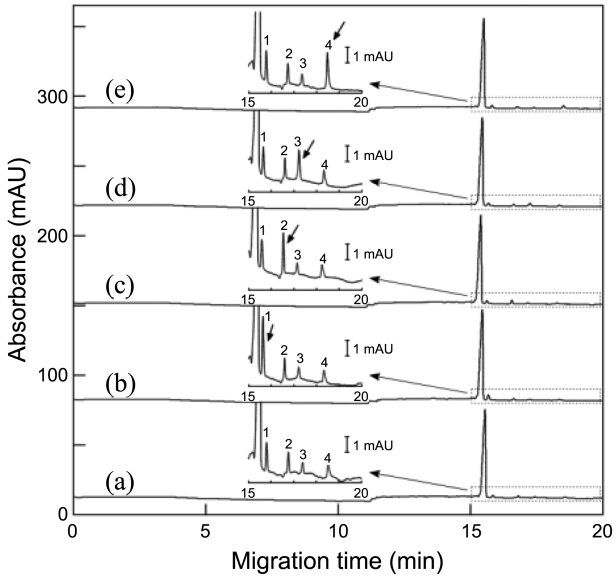


**Figure 4.** Electropherograms of trace metal ions in human urine. (a) The urine sample was 1:20 diluted with 250 mM NaCl containing 500 nM DCF, $Ni^{2+}$, and $Cd^{2+}$. The diluted urine sample additionally spiked with (b) 500 nM DCF, (c) 500 nM $Ni^{2+}$, (d) 500 nM $Zn^{2+}$, and (e) 500 nM $Cd^{2+}$. Peaks: 1; DCF, 2; Ni-TAR, 3; Zn-TAR, and 4; Cd-TAR. Injection: 160 s at $3 \times 10^3$ Pa. Other conditions as in Figure 3.

## Conclusions

Trace metal ions in a highly saline matrix were sensitively

**Table 1.** LOD, linear correlation coefficients ($r$), calibration equation, dynamic ranges, and efficiencies

| | LOD[a] | $r$ | Regression line[b] | Dynamic range | Efficiency[c] |
|---|---|---|---|---|---|
| Ni-TAR | 17 nM | 0.997 | $x = (y + 1.874)/0.191$ | 17-14000 nM | $5.2 \times 10^5$ |
| Zn-TAR | 15 nM | 0.997 | $x = (y - 3.576)/0.194$ | 15-15000 nM | $2.2 \times 10^5$ |
| Cd-TAR | 27 nM | 0.998 | $x = (y + 1.981)/0.375$ | 44-8900 nM | $3.0 \times 10^5$ |

[a]Sample in 250 mM NaCl injected for 420 s at $3 \times 10^3$ Pa. [b]$y$ = Peak area $\times 10^3$ (mAU s), $x$ = concentration (nM). [c]The average of 4 results from 1 $\mu$M $Ni^{2+}$, $Zn^{2+}$, and $Cd^{2+}$

**Table 2.** Analysis of the reference material for urine, ME 28351

| | Assigned values | | Obtained values |
|---|---|---|---|
| | Assigned concentration | Confidence range (Double standard deviation) | Concentration by our method |
| Ni | 410 nM | 310-510 nM | 430 ± 20 nM[a] |
| Zn | 26000 nM | 20000-32000 nM | 28000 ± 1000 nM[a] |
| Cd | 110 nM | 90-140 nM | 90 ± 20 nM[a] |

[a]Standard deviation of four aliquots analyzed sequentially.

determined by IAOC with TAR monitoring absorbance at 500 nm. A highly concentrated zone of TAR from the BGE under TITP conditions, with $Cl^-$ in the sample as the LE and $CHES^-$ in the BGE as the TE, was formed at the rear of the sample matrix. The metal cations migrating toward the cathode in the sample matrix were subsequently swept by the isotachophoretically stacked TAR by on-line complexation forming anionic metal-TAR which were also stacked under the same TITP conditions. Our IAOC scheme synergistically combines the advantages of the derivatizing of metal ions with a chromophore, the stacking of a large volume of sample, and convenient on-line complexation without complicated pretreatment steps such as desalting. The LODs ($S/N = 3$) were 17 nM for $Ni^{2+}$, 15 nM for $Zn^{2+}$, and 27 nM for $Cd^{2+}$ using the standard absorbance detection system of a commercial CE instrument. The LOD values can be lowered even further simply with a longer sample injection using a longer capillary.

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