

Full Length Research Paper

Analysis of arsenic species in realgar bioleaching solution by capillary zone electrophoresis

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Received 30 March, 2013; Accepted 1 December, 2014

Realgar was bioleached by *Acidithiobacillus ferrooxidans* BY-3. A capillary zone electrophoresis (CZE) method was developed to analyze arsenic species in realgar bioleaching solution. This way can simultaneously separate arsenic compounds including arsenite (iAs^{III}), arsenate (iAs^V), monomethylarsenic (MMA^V) and dimethylarsenic acid (DMA^V). Borate and cetyltrimethylammonium bromide (CTAB) were selected to compose a background electrolyte (BGE). Identification and quantification of the arsenic species at $\mu\text{g/ml}$ levels was possible by use of direct UV detection at 195 nm. The limits of detection and quantification for the targeted analytes were in the range of 3.10–12.00 and 10.34–39.83 $\mu\text{g/ml}$, respectively. The intraday precision was in the range of 1.45–2.06% for migration times and 3.14–4.28% for peak areas, while interday precision was in the range of 2.24–3.20% for migration times and 6.46–7.11% for peak areas, respectively. Spiked recoveries at three levels were in the range of 84.92–101.67%. Results indicated that the bioleaching solution of realgar only exists in organic arsenic, the components were identified to be arsenite and arsenate.

Key words: Realgar, bioleaching, *Acidithiobacillus ferrooxidans*, arsenic species, capillary zone electrophoresis.

INTRODUCTION

Realgar, also called red arsenic or *Xiong-Huang* (China), has poor solubility in water. It contains more than 90% of arsenic disulfide (As_2S_2) or tetra-arsenic tetra-sulfide (As_4S_4) and small quantities of arsenic trioxide (As_2O_3) (Liu et al., 2008). The medical use of realgar has been traced back to thousands of years ago for various diseases. In recent years, realgar was believed to have antibiotic, anti-viral and anti-tumor effects (Wang et al., 2008; Xu et al., 2006). Notably, realgar has been effectively applied to treat chronic myelocytic leukemia

(CML) and acute promyelocytic leukemia (APL) (Zhang et al., 2008).

Realgar was extracted using several methods to improve clinical efficacy. For example, realgar can be extracted with elutriation method and alkali solvent before clinical use (National Pharmacopoeia, 2005; Wu et al., 2004). With the increase of solubility, the toxicity will increase while the efficiencies of extracts will be low. Therefore, we used bioleaching technologies to deal with medicinal realgar so as to obtain a new reconstituent

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Table 1. Arsenic compound of interest.

Chemicals with symbolic name	Structure	$\lambda_{\max}(\text{nm})$	pKa
MMA ^V	$\begin{array}{c} \text{OH} \\ \\ \text{O}=\text{As}-\text{ONa} \\ \\ \text{CH}_3 \end{array}$	192	3.6 8.2
DMA ^V	$\begin{array}{c} \text{O} \\ \\ \text{H}_3\text{C}-\text{As}-\text{CH}_3 \\ \\ \text{OH} \end{array}$	194	6.2 9.3
iAs ^{III}	$\begin{array}{c} \text{OH} \\ / \\ \text{HO}-\text{As} \\ \backslash \\ \text{ONa} \end{array}$	197	9.2 13.5
iAs ^V	$\begin{array}{c} \text{OH} \\ \\ \text{KO}-\text{As}-\text{OH} \\ \\ \text{O} \end{array}$	192	2.3 6.9 11.4

which is expected to possess increased efficiency and decreased toxicity.

Bioleaching is a process for dissolution of metals from their mineral source through the use of bacteria to convert insoluble metal sulfides (CoS, PbS and ZnS) into water-soluble metal sulfates (CoSO₄, PbSO₄ and ZnSO₄). Realgar is a mineral containing both sulfur and arsenic, and it has been reported that arsenic was bioleached from realgar (Zhang et al., 2007). Although realgar bioleaching solution had better therapeutic effect than arsenic trioxide for hepatoma, lung cancer and leukocytopenia *in vivo/vitro* by inducing apoptosis, the active substances had not been identified (Zhang et al., 2008). We expect that the bacterial realgar bioleaching solution contain iAs^{III} species and methylated arsenic species.

For the elucidation of active substances in realgar bioleaching solution, different arsenic compounds were analyzed in a recent study. It has been reported that high-performance liquid chromatography (HPLC) with a variety of detection methods including inductively coupled plasma mass spectrometry (ICP-MS), hydride generation atomic fluorescence spectrometry (HG-AFS) (Ammann, 2011; Campillo et al., 2008; Cui et al., 2004; Gonzalez-Contreras et al., 2011; Hata et al., 2007; Jaafar et al., 2007; Kirby et al., 2004; Raab et al., 2004; Tlustos et al., 2004; Yang et al., 2012; Zhao et al., 2011), hydride generation inductively coupled plasma mass spectrometry (HG-ICPMS) can be used for separating arsenic compounds (Inoue et al., 1999). Compared with HPLC, capillary electrophoresis has been demonstrated to be a simple and powerful separation technique for arsenic. And it has advantages of many separation modes, high selectivity and sensitivity, small quantities

and doses of reagent and sample dosage.

The aim of this work was to develop a rapid CZE method for the simultaneous determination of inorganic and organic arsenic compounds including iAs^{III}, iAs^V, MMA^V and DMA^V, and was validated to determine parameters such as linearity, detection and quantification limit, precision, recovery and stability. The method was also successfully applied to analyze the arsenic species from leachate by bioleaching of realgar using *Acidithiobacillus ferrooxidans* BY-3.

MATERIALS AND METHODS

Realgar and arsenic standards

Realgar (As₂S₂, 99.1% in purity), was obtained from Shimen County, Hunan Province, China. Disodium tetraborate decahydrate (≥98% in purity) was purchased from Tianjin Chemical Reagent Factory (China). Dimethylarsenic acid [(CH₃)₂AsO(OH), DMA^V] and potassium arsenate (KH₂AsO₄, iAs^V) were purchased from Sigma Co. Ltd. (America). Sodium monomethyl-arsenate (CH₃AsNaO₃·(3/2)H₂O, MMA^V) was purchased from Shanghai Quanda Scientific Trade Co. Ltd. (China). Arsenic trioxide (As₂O₃) was purchased from Beijing Chemical Reagent Factory (China). All other reagents were of analytical or HPLC grade. Arsenic sample solutions were prepared by dissolving in Milli-Q water to form the stock solutions with concentrations of 1 mg/ml. Before storage, all stock solutions were filtered through a 0.22 μm nylon filter membrane and degassed in ultrasonic bath for 15 min. The molecular structures, maximum UV absorbance and pKa values of the arsenic analytes are shown in Table 1 (Sun et al., 2004).

Bioleaching experiments and preparation of realgar bioleaching solution

Bioleaching of realgar was carried out in 500 ml flasks with 200 ml

of the 9 K medium (with 1 g of sterile sulfur powder per liter) containing 0.2 g of realgar powder sample with an initial pH of 2.0 (Kutschera et al., 2007). Each flask was inoculated with *A. ferrooxidans* BY-3 (CCTCC- M203071) suspension at 10% (v/v). The flasks were incubated at 150 rpm, 28°C, with a pH of 2.0. The experiment lasted for 30 days.

The bacterial cultures were centrifuged at 2000 rpm for 15 min to remove the precipitation, and then the supernatant were filtered through a 0.22 µm nylon filter membrane. The filtrate is the realgar bioleaching solution, and injected directly after degassing by sonication.

Instruments and conditions

All CZE separations were performed on a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA, USA). This system was equipped with a reversible-polarity power supply (0 to (±30) kV) and a photo-diode-array detector. The system was controlled by a 32 Karat system and version 5.0 for data collection and analysis. The separations were carried out on an uncoated fused-silica capillary (60 cm × 75 µm i.d.) with a detection window located 10 cm from its extremity. A built-in temperature control system was designed to maintain separation temperature and minimize Joule heat generated, thus enabling the CZE system to use a high electric field and to achieve very low band dispersion. The detection wavelength was set at 195 nm. The separation voltage was set at -20 kV and the optimized buffer was composed of 20 mM borate/0.5 mM CTAB at pH 9.5 by titrating with 0.1 M NaOH. The BGE was prepared daily and vortexed for 2 min, filtered through 0.45 µm filter membrane and degassed for 15 min. Before first use, a new capillary was rinsed with 1 M HCl for 5 min, followed by water for 2 min, 0.1 M NaOH for 10 min, water for 2 min and running buffer for 15 min. Samples were injected by applying a pressure of 0.5 psi for 5 s (1 psi = 6894.76 Pa).

RESULTS AND DISCUSSION

Development of capillary zone electrophoresis

Effect of the borate concentration

In general, ion migration velocity, separation, column efficiency and peak shape in CZE are sensitive to changes in BGE characteristics. In this paper, to verify the effect of buffer concentration on migration behavior, the running buffer containing 0.5 mM CTAB and borate with concentrations from 20 to 50 mM at pH 9.5, applied voltage of -17.5 kV was investigated to separate arsenic species. Figure 1 shows the effects of different borate concentrations on the electrophoretic separation of arsenic species. Obviously, retention time, peak height and peak area (sensitivity) were affected by the borate concentration. With increasing borate concentration, migration became slower. This may be due to the higher Joule heating caused by increased conductivity of the buffer and decreases in electroosmotic flow (EOF), zeta-potential and ion migration velocity at the capillary wall-solution interface with increasing ion strength. Considering the Joule heats, migration times, column efficiency, peak shape and resolution of real samples, 20 mM borate containing 0.5 mM CTAB was selected as BGE.

Effect of the buffer pH value

The electrophoretic mobility of the arsenic compounds is strongly dependent on their pKa value as shown in Table 1 and the pH of the running electrolyte solution since arsenic compounds contain acidic groups in their chemical structure. This fact is especially important in the case of arsenic compounds showing several pKa values. These arsenic compounds can be present in different ionized forms depending on the pH of buffer, and therefore it is possible to change their electrophoretic mobility. The effects of different BGE pH values from 9.0 to 10.5 with a 0.5 (±0.02) -step on the electrophoretic separations of arsenic species is shown in Figure 2. When pH value was below 9.5, the baseline separation of anions including iAs^{V2-} , MMA^{2-} , DMA^{2-} , iAs^{III2-} was achieved, however, the detection sensitivity was lower than that at pH 9.5; when pH value was above 9.5, iAs^{V2-} , iAs^{III2-} had lower sensitivity than that at pH 9.5; when pH value was above 10.0, MMA^{2-} , DMA^{2-} and iAs^{V2-} had low separation and sensitivity; Whole migration became much slower with increase of the BGE pH because pH alters the charges and thus electrophoretic mobilities. Although in the above pH range each arsenic analyte should be at the same ionization degree: iAs^{V2-} , MMA^{2-} , DMA^{2-} and iAs^{III2-} , migration profiles were different. The migration order was iAs^{V2-} , MMA^{2-} , DMA^{2-} and iAs^{III2-} since the EOF increases with increase zeta potential. Furthermore, the zeta potential is directly proportional to charge-mass ratio (charge-mass ratio: $iAs^{III2-} > DMA^{2-} > MMA^{2-} > iAs^{V2-}$). In contrast, satisfactory separation and sensitivity were obtained at pH 9.5, indicating that the buffer capacity was high enough that local pH and conductivity did not change as a result of sample injection and separation. Finally, a BGE of pH 9.5 was selected.

Effect of separation voltage

The inorganic and organic arsenic species investigated are acidic compounds which are not totally dissociated under normal conditions. The dissociation degree of the species, which affects ionic charge and electrophoretic mobility, depends on the pH of the running electrolyte solution and the pKa of the species. Under normal conditions (the capillary surface is negatively charged and positive voltage is applied) the EOF transports, firstly, the undissociated forms of the arsenic compounds toward the cathode and the partially dissociated anionic compounds then follow. In the electropherograms, only anionic species with lower electrophoretic mobilities than the magnitude of the EOF can be observed. Although, increasing the buffer pH results in increased electroosmotic mobility, the electrophoretic mobilities of the arsenic compounds also increase, because the compounds are more dissociated. A complete separation is obtained by use of a negative voltage, and the direction of the EOF can be reversed. In this case, the electrophoretic

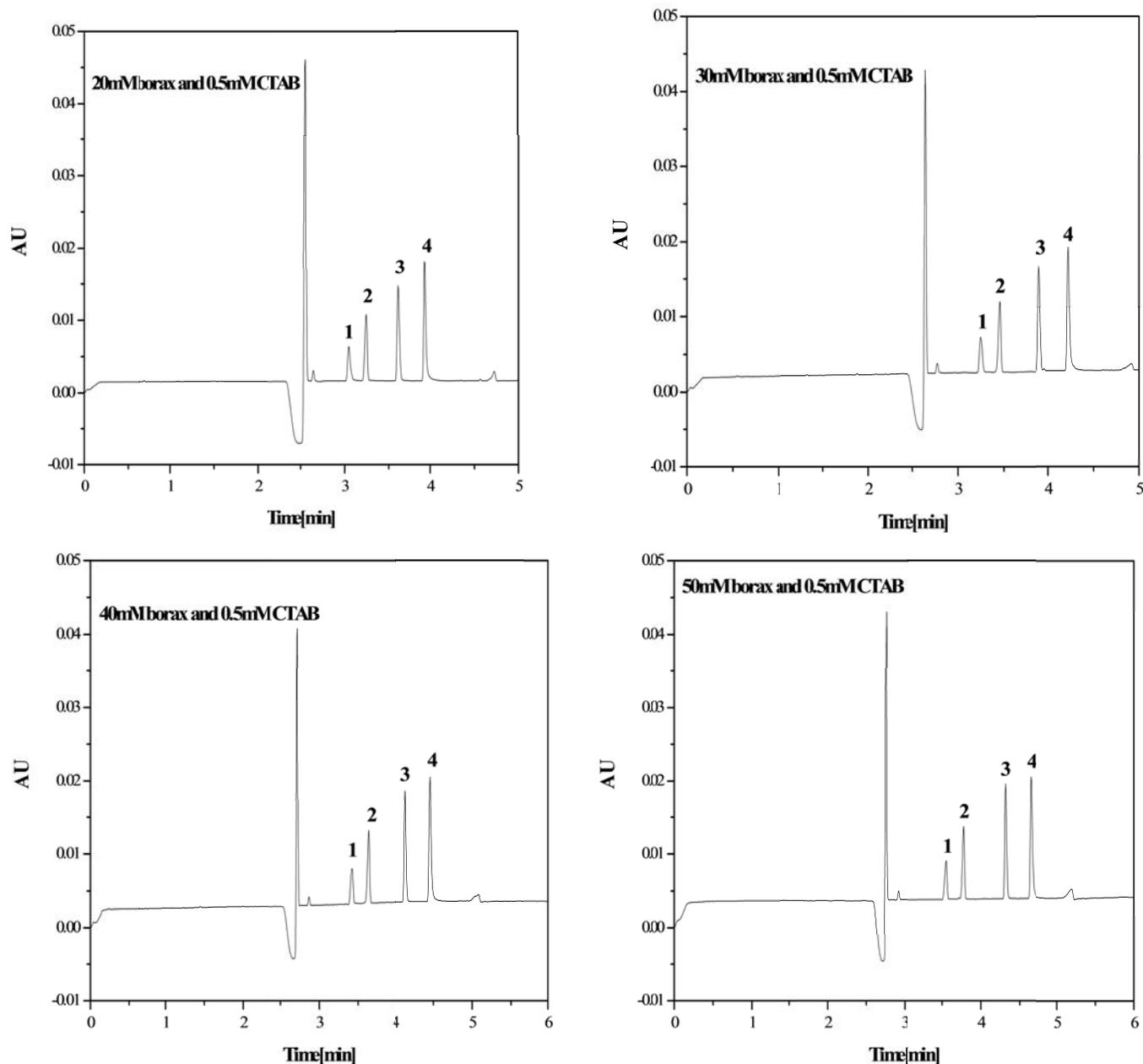


Figure 1. Electropherogram obtained from the four arsenic compounds at different borate concentration. sample, 250 $\mu\text{g/ml}$ $\text{iAs}^{\text{V}2-}$, 250 $\mu\text{g/ml}$ MMA^{2-} , 250 $\mu\text{g/ml}$ DMA^{2-} , 250 $\mu\text{g/ml}$ $\text{iAs}^{\text{III}2-}$; buffer pH 9.5; injection pressure, 0.5 psi for 5 s; detection wavelength, 195 nm; voltage, -17.5 kV; temperature, 25°C; Peaks: (1) $\text{iAs}^{\text{V}2-}$; (2) MMA^{2-} ; (3) DMA^{2-} ; (4) $\text{iAs}^{\text{III}2-}$.

and electroosmotic mobilities are oriented in the same direction-towards the anode, reversal of the EOF was achieved by addition of 0.5 mM CTAB to the running buffer. The effect of applied negative voltage on migration times and separation efficiencies was also investigated with separations in the voltage range from -20 to -15 kV with a 2.5-step at pH 9.5. Figure 3 shows that the migration times decreased but the detection sensitivity did not improve with increasing voltage while the baseline noise decreased. This may be attributed to the higher

Joule heating and lower viscosity of the buffer with increasing voltage. Thus -20 kV was selected as the separation voltage.

Choice of detection wavelength

The iAs^{III} , iAs^{V} , MMA^{V} and DMA^{V} have absorption maxima at wavelengths between 190 and 205 nm (Table 1). These species can be detected by direct UV detection,

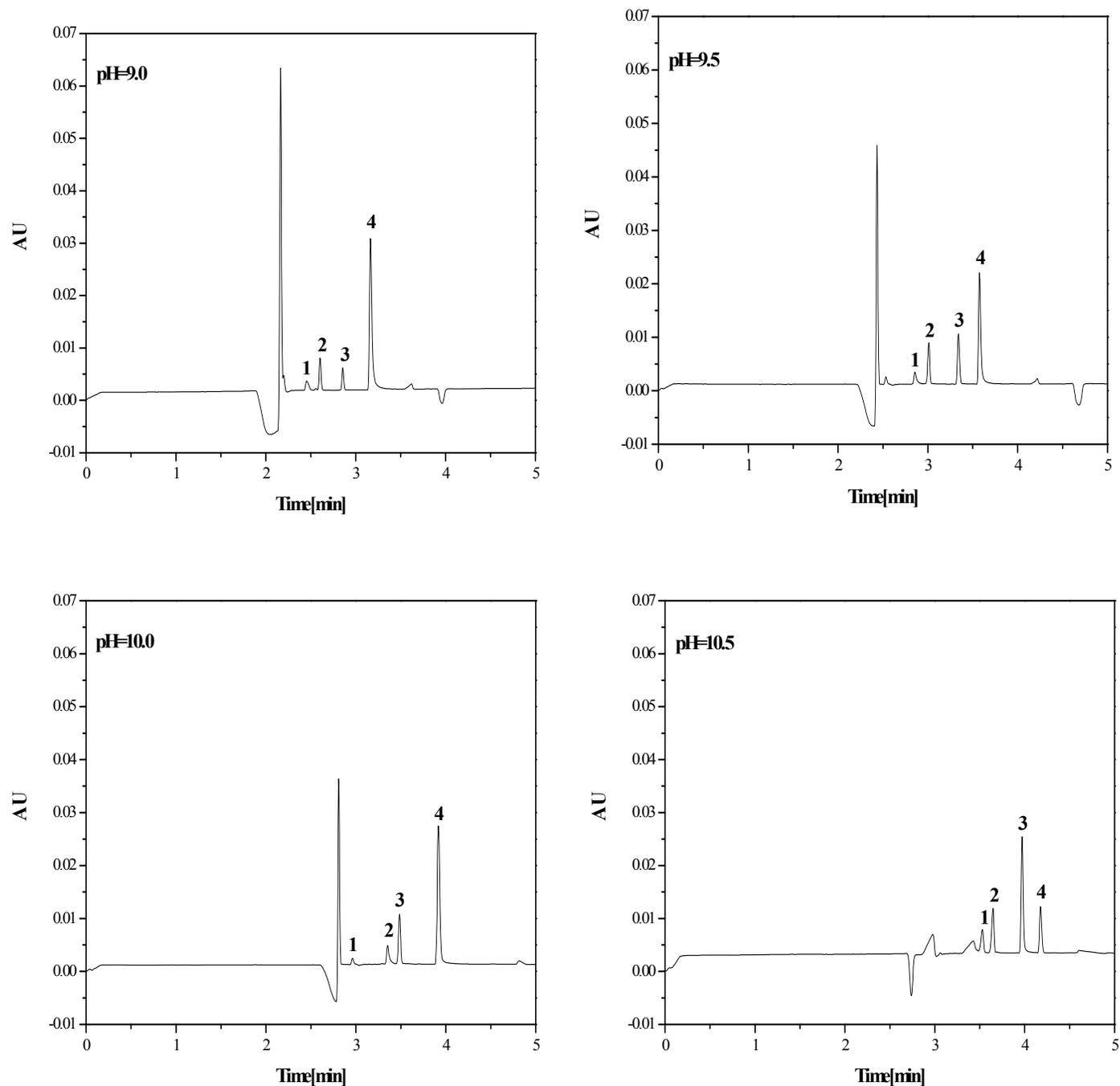


Figure 2. The effects of BGE pH on the electrophoretic separation of arsenic compounds. Buffer, 20 mM borate, containing 0.5 mM CTAB; Sample, 250 $\mu\text{g/ml}$ $\text{iAs}^{\text{V}2-}$, 250 $\mu\text{g/ml}$ MMA^{2-} , 250 $\mu\text{g/ml}$ DMA^{2-} , 250 $\mu\text{g/ml}$ $\text{iAs}^{\text{III}2-}$; injection pressure, 0.5 psi for 5 s; detection wavelength, 195 nm; voltage, -17.5 kV; temperature, 25°C; Peaks: (1) $\text{iAs}^{\text{V}2-}$; (2) MMA^{2-} ; (3) DMA^{2-} ; (4) $\text{iAs}^{\text{III}2-}$.

because solutions of borate buffer are more UV transparent. To select an optimum detection wavelength, a wavelength range of 195-205 nm was tested. Figure 4 shows the electropherograms of $\text{iAs}^{\text{V}2-}$, MMA^{2-} , DMA^{2-} and $\text{iAs}^{\text{III}2-}$ obtained using 20 mM borate, pH 9.5 and containing 0.5 mM CTAB at all tested detection wavelengths. With increase in the detection wavelength

from 195 to 205 nm, the sensitivity of detection decreased while the baseline noise increased. After considering the detection sensitivity and baseline noise, 195 nm was thus selected as the detection wavelength.

In BGE of pH 9.5, consisting of 20 mM borate and 0.5 mM CTAB, the separation condition were 195 nm detection wavelength, injection pressure 0.5 psi for 5 s,

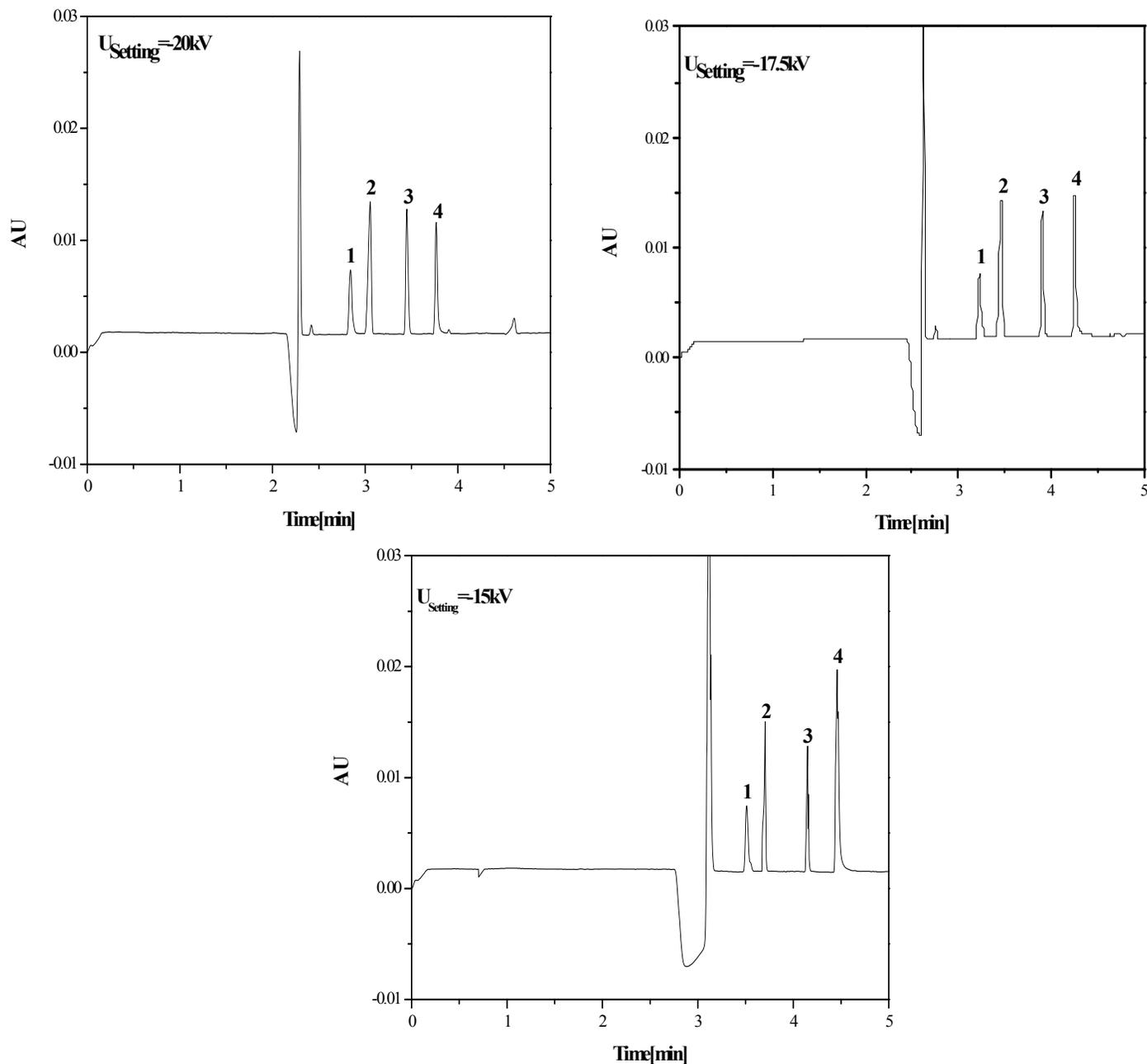


Figure 3. The electropherograms was obtained at different voltage. Buffer, 20 mM borate and 0.5 mM CTAB; pH 9.5; Sample, 576 $\mu\text{g/ml}$ $\text{iAs}^{\text{V}2-}$, 192 $\mu\text{g/ml}$ MMA^{2-} , 192 $\mu\text{g/ml}$ DMA^{2-} , 40 $\mu\text{g/ml}$ $\text{iAs}^{\text{III}2-}$; injection pressure, 0.5 psi for 5 s; temperature, 25°C. Peaks: (1) $\text{iAs}^{\text{V}2-}$; (2) MMA^{2-} ; (3) DMA^{2-} ; (4) $\text{iAs}^{\text{III}2-}$.

and -20 kV separation voltage, a baseline level resolution of four arsenic compounds was achieved (Figure 5).

Calibration curve and validation of the method

With 20 mM borate and 0.5 mM CTAB BGE at pH 9.5, the calibration curves were calculated by analyzing the $\text{iAs}^{\text{V}2-}$, MMA^{2-} , DMA^{2-} and $\text{iAs}^{\text{III}2-}$ as $y = a+bx$, where x was peak

area, and y was the concentration. Five concentration points were used to prepare the calibration curve for each compound. The linear regression equation of the calibration curve and correlation coefficient (r) are shown in Table 2. The limit of detection (LOD) is the lowest concentration of the analyte in a sample that can be detected, equal to 3 s , where s is the sample standard deviation of 10 independent sample blanks fortified at lower concentrations, measured one each. The limit of

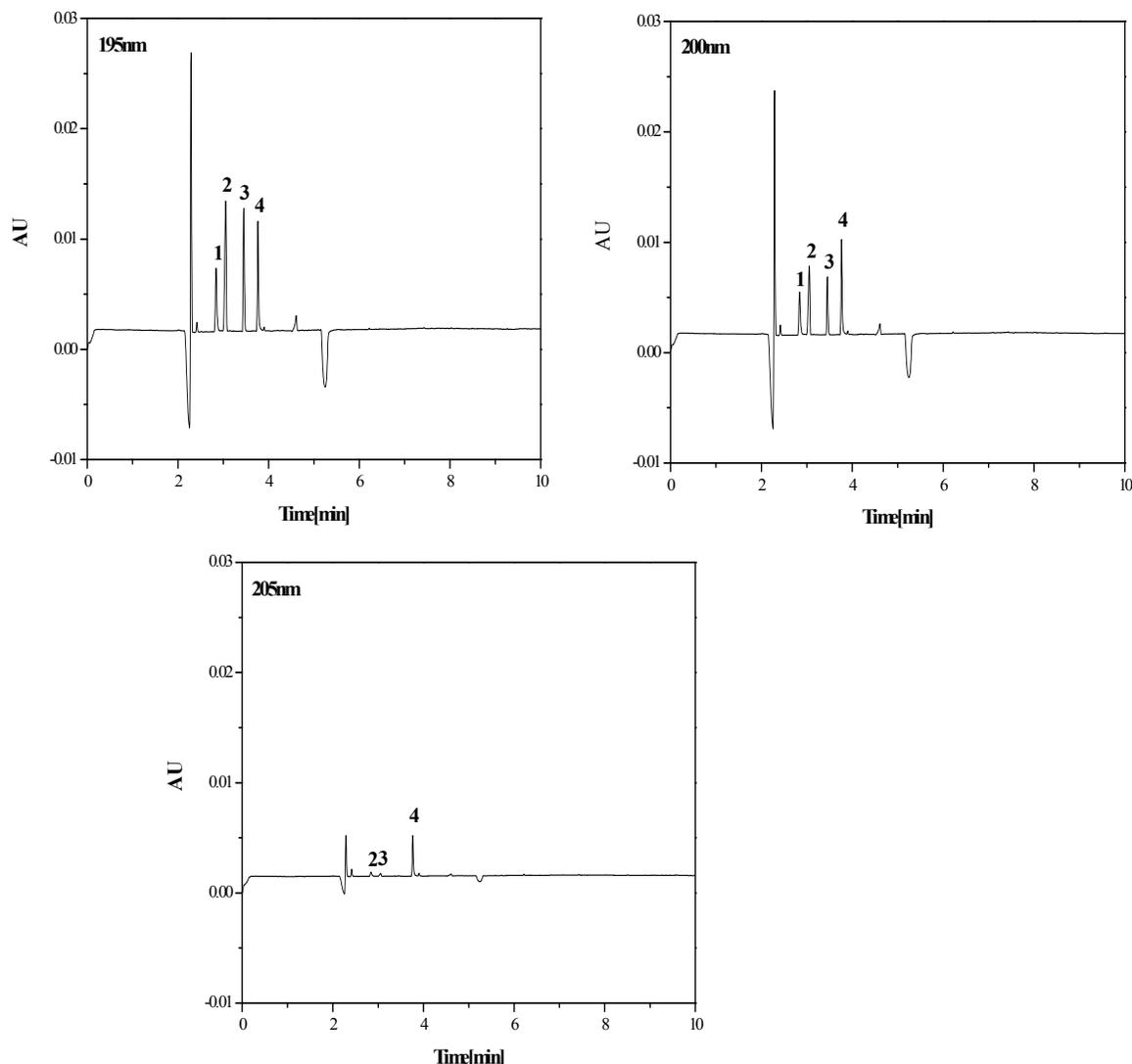


Figure 4. The electropherograms was obtained at different detection wavelengths. Buffer, 20 mM borate, pH 9.5, containing 0.5 mM CTAB; Sample, 650 $\mu\text{g/ml}$ $\text{iAs}^{\text{V}2-}$, 160 $\mu\text{g/ml}$ DMA^{2-} , 160 $\mu\text{g/ml}$ MMA^{2-} , 30 $\mu\text{g/ml}$ $\text{iAs}^{\text{III}2-}$; injection pressure, 0.5 psi for 5 s; voltage, -20 kV; temperature, 25°C. Peaks: (1) $\text{iAs}^{\text{V}2-}$; (2) MMA^{2-} ; (3) DMA^{2-} ; (4) $\text{iAs}^{\text{III}2-}$.

quantification (LOQ) is the concentration of the fortified sample that can be quantified with less than 20% variation in precision, according to this rule, LOD and LOQ were also displayed in Table 2. Table 3 shows that the intraday precision expressed as relative standard deviations (R.S.D.%) with respect to migration time and peak area from five successive injections for the different analytes was found to be 1.45-2.06% for migration times and 3.14 - 4.28% for peak areas. The interday precision found by analyzing different analytes on three successive days was found to be 2.24 - 3.20% for migration times and around 6.46 -7.11% for peak areas. The deviation of the retention time observed in the intraday tests was lower than the deviation observed in the interday tests. This is logical, since small changes when the running

buffer is prepared can affect the retention time of the analytes. The spiked recoveries were 84.92, 96.88, 101.67 and 88.65% for iAs^{V} , DMA^{V} , MMA^{V} and iAs^{III} .

Identification of arsenic species in the realgar bioleaching solution

In the first phase of bioleaching, the powder of realgar was insoluble in culture. After 15 days, a portion realgar was soluble and the precipitation was observed in the flask. After 30 days, the realgar powders were all soluble. Then the cultures were used for preparation of realgar bioleaching solution.

It has been reported that the main components in the

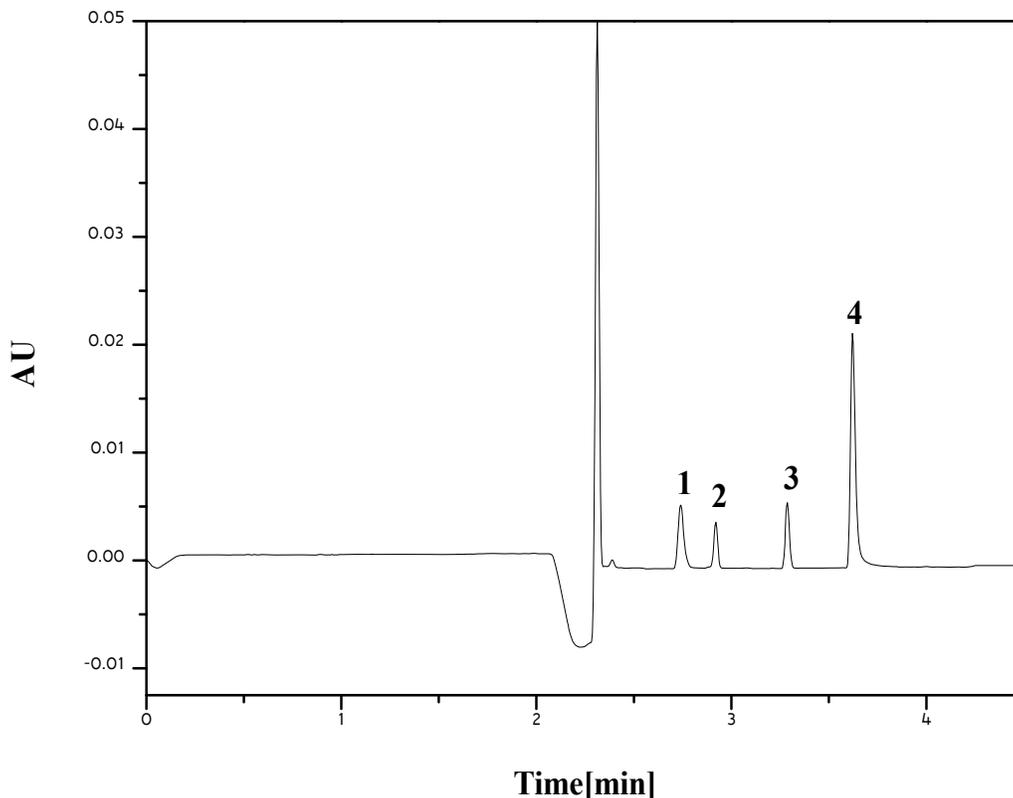


Figure 5. The electrophoregrams of arsenic compounds from standard mixture. Buffer, 20 mM borate, pH 9.5, containing 0.5 mM CTAB; Sample, 500 $\mu\text{g/ml}$ $\text{iAs}^{\text{V}2-}$, 120 $\mu\text{g/ml}$ MMA^{2-} , 130 $\mu\text{g/ml}$ DMA^{2-} , 250 $\mu\text{g/ml}$ $\text{iAs}^{\text{III}2-}$; injection pressure, 0.5 psi for 5 s; voltage, -20 kV; temperature, 25°C. Peaks: (1) $\text{iAs}^{\text{V}2-}$; (2) MMA^{2-} ; (3) DMA^{2-} ; (4) $\text{iAs}^{\text{III}2-}$.

Table 2. Linearity range ($\mu\text{g/ml}$), calibration line and detection limits for arsenic compounds.

Analyte	Linearity range ($\mu\text{g/ml}$)	Calibration line		Detection limits	
		Regression equation ^a	r	LOD ($\mu\text{g/ml}$) ^b	LOQ ($\mu\text{g/ml}$) ^b
iAs^{V}	50-500	$y = 0.0412x + 4.3121$	0.99982	12.00	39.83
MMA^{V}	12-120	$y = 0.0378x + 4.4839$	0.99975	3.13	10.46
DMA^{V}	13-130	$y = 0.0155x - 4.7362$	0.99930	3.88	12.94
iAs^{III}	25-250	$y = 0.0027x - 2.473$	0.99972	3.10	10.34

a. y and x stand for the peak area and the concentration ($\mu\text{g/ml}$) of the analytes, respectively. b. The LOD was defined as the concentration where the signal-to-noise ratio is 3 and the LOQ was defined as the concentration where the signal-to-noise ratio is 5.

Table 3. Precision and recovery of the analytical method.

Analyte	Intraday, R.S.D. (%) (n=5)		Inter-day, R.S.D. (%) (n=5)		Recovery, R.S.D. (%) (n=5)
	Migration time	Peak area	Migration time	Peak area	
iAs^{V}	1.45	3.66	2.24	6.46	84.92
MMA^{V}	1.61	4.28	2.54	6.57	96.88
DMA^{V}	1.87	4.27	2.76	6.74	101.67
iAs^{III}	2.06	3.14	3.20	7.11	88.65

alkali extracts of realgar were $\text{iAs}^{\text{III}2-}$ and $\text{iAs}^{\text{V}2-}$ (Wu et al., 2004). However, the determination of inorganic and

organic analytes in real samples by CZE with direct ultraviolet detection was challenging since the

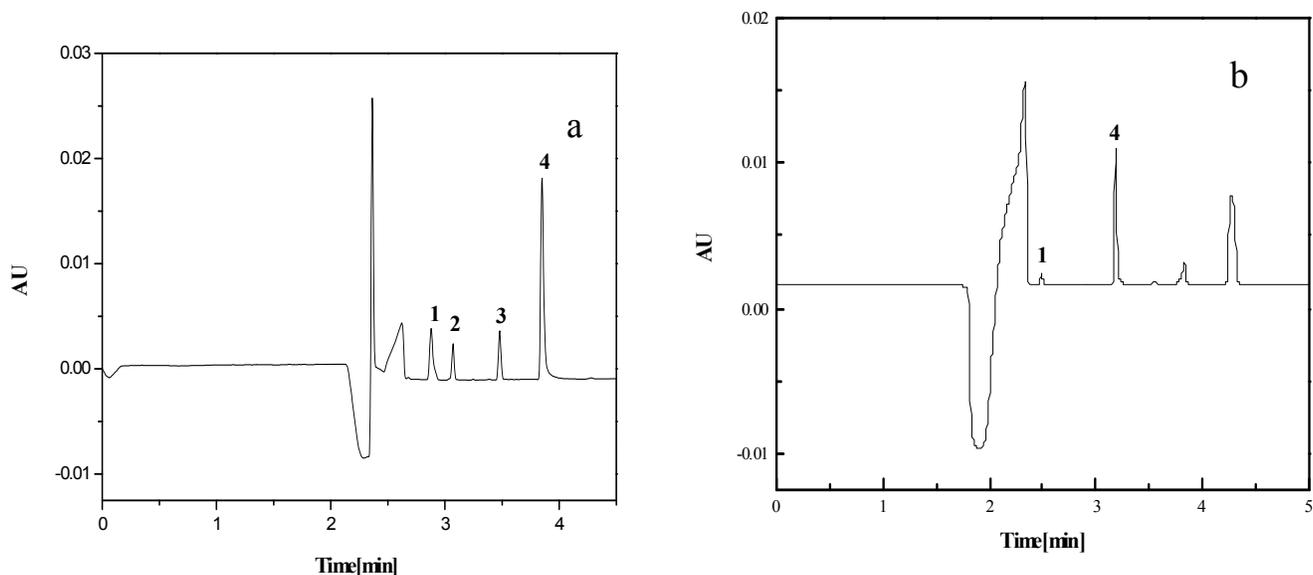


Figure 6. The electrophoregrams of (a) realgar bioleaching solution by adding appropriate standard, and (b) bio-leachate of realgar. Buffer, 20 mM borate and 0.5 mM CTAB; pH 9.5; injection pressure, 0.5 psi for 5 s; detection wavelength, 195 nm; voltage, -20 kV; temperature, 25°C. Peaks: (1) iAs^{V2-} ; (2) MMA^{2-} ; (3) DMA^{2-} ; (4) iAs^{III2-} .

components of realgar bioleaching solution were unknown. The realgar bioleaching solution was analyzed using the established CZE method, the corresponding electrophoregrams indicated that iAs^{V2-} and iAs^{III2-} were the main components. Figure 6 shows that no signal peak was detected except iAs^{V2-} and iAs^{III2-} when compared with the electrophoregrams of the realgar bioleaching solution by adding appropriate standards. Using the established calibration curves, the concentrations of iAs^{V2-} and iAs^{III2-} in realgar bioleaching solution were 102.28 and 136.36 $\mu\text{g/ml}$ as As. So far, this work is the first report on using CZE method with direct UV BGE to analyze the realgar bioleaching solution, adding valuable information for studying the components in the bioleaching of realgar.

Conclusion

A CZE method was developed and used to analyze arsenic species in realgar bioleaching solution produced by *A. ferrooxidans* BY-3. The CZE with direct UV detection showed excellent suitability for the simultaneous separation and determination of the inorganic and organic arsenic compounds using the borate as BGE. The reliability of the technique has also been verified by analysis of linearity, repeatability, reproducibility and sensitivity. The arsenic species in realgar bioleaching solution produced by *A. ferrooxidans* were iAs^{V2-} and iAs^{III2-} , and their concentrations were found to be 102.28 and 136.36 $\mu\text{g/ml}$, respectively.

Conflict of Interest

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by Natural Science Foundation of Heilongjiang Province of China (QC2014C023). The authors are very grateful to Mr. Yuming Dong (Lanzhou University, China) and Mrs. Lifang Zheng (Lanzhou University, China) for their technical assistance in CZE measurement, and to Xu Zhang (Lanzhou University, China) for his help in obtaining the realgar bioleaching solution.

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