An Investigation on Heavy Metals Bio-Sorption by *Azolla filiculoides* in the International Anzali Wetland

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ABSTRACT: The ultrasound assisted emulsification microextraction (USAEME) method followed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) was used for preconcentration and determination of bismuth (Bi), indium (In) and lead (Pb) in the plant *Azolla filiculoides* Lam. Calix [4] pyrrole was used as a chelating agent and tetrachloroethylene was selected as extraction solvent. The main parameters of the USAEME method were optimized by using response surface methodology (RSM). The optimal conditions were as follows: 130 μL for volume of extraction solvent, 4 min for sonication time, 6.75 (w/v %) for salt concentration, 890 mg/L for concentration of chelating agent and 7.5 for pH. The calibration graphs were linear in the range of 1-1500 μg/L for Bi and In, and 1-1000 μg/L for Pb with the determination coefficients (R^2) of 0.9983, 0.9974 and 0.9997, respectively. The limits of detection (LODs) for Bi, In and Pb were 0.57, 0.76 and 0.84 μg/L, respectively. Under the optimal conditions, the method was successfully applied to the determination of Bi, In and Pb in *Azolla filiculoides* Lam. tissues in Anzali wetland and satisfactory relative recoveries (94–97%) were achieved.

Key words: Heavy metals, Anzali wetland, *Azolla filiculoides,* Experimental design, ICP-AES

INTRODUCTION

Heavy metals are considered to be one of the main sources of pollution in the environment, since they have a significant effect on the ecological quality (Sastre, *et al.*, 2002). Excessive levels of heavy metals can be introduced into the environment as a consequence of human activities and rapid industrialization (Sereshti, *et al.*, 2012). The plants in or close to contaminated sites can uptake and accumulate these metals, and then exert potential risk to humans and animals (Fu, *et al.*, 2008). The major route for heavy metals exposure to humans is mainly through soil–crop–food pathway (Fu, *et al.*, 2008). Consumption of food crops contaminated with heavy metals is a major food chain route of human exposure.

Anzali wetland located in the south-west of Caspian sea (Guilan province north of Iran), is one of the important natural habitat of a wide variety living things. Heavy metals are one of the main sources of water pollution in this ecosystem and become the greatest risk to the animals and plants life. Azolla *filiculoides* Lam. is a floating aquatic fern, which is widely distributed in Anzali wetland. This plant has been used as a green manure to improve soil fertility and rice production. It has been reported that Azolla has a high capacity to accumulate heavy metals from aqueous media (Zhang, *et al.*, 2008; Sánchez-Viveros, *et al.*, 2010). A literature review showed that the biosorption of several heavy metals such as cesium (Cs) , strontium (Sr) (Ghorbanzadeh Mashkani and Tajer Mohammad Ghazvini., 2009), cadmium (Cd) (Rakhshaee, *et al.*, 2006; Khosravi, *et al.*, 2005; Ganji, *et al.*, 2005), lead (Pb) (Rakhshaee, *et al.*, 2006; Khosravi, *et al.*, 2005; Ganji, *et al.*, 2005), nickel (Ni) (Rakhshaee, *et al.*, 2006; Khosravi, *et al.*, 2005), zinc (Zn) (Rakhshaee, *et al.*, 2006; Khosravi, *et al.*, 2005; Zhao, *et al.*, 1999; Ganji, *et al.*, 2005), and copper (Cu) (Zhao, *et al.*, 1999; Ganji, *et al.*, 2005) by Azolla has been investigated with various analysis techniques. The monitoring of the heavy metals in environmental samples is an important part of analytical chemistry due to their positive and/or negative influences on

the human body (Soylak and Tuzen, 2008). A variety of analysis techniques such as flame atomic absorption spectrometry (FAAS) (Schwingel Ribeiro, *et al.*, 2013; Shah, *et al.*, 2011; Citak and Tuzen, 2010; Candir, *et al.*, 2008), ICP-AES (Sereshti, *et al.*, 2012; Sun and Wu, 2011; Kagaya, *et al.*, 2005), inductively coupled-plasma mass spectrometry (ICP-MS) (Yilmaz , *et al.,* 2012), atomic fluorescence spectrometry (AFS) (Zhou, *et al.*, 2011; Liu, *et al.*, 2008) , graphite furnace-atomic absorption spectrometry (GF-AAS) (Tokman, *et al.*, 2003; Minagawa, *et al.*, 1993; Acar, *et al.*, 2000), ion chromatography (IC) (Sutton, *et al.*, 1997), UV–Visible spectrophotometry (Gumus, *et al.*, 2005), voltammetry (Kokkinos , *et al.*, 2012; Rao and Koshy, 1993), potentiometry (Teixeira and Fatibello-Filho, 2001), amperometry (Vydra and Vorlíček, 1966), chronoamperometry (Yasri, *et al.*, 2011) and coulometry (Timofeeva, *et al.*, 2011) have been used for determination of Bi, In and Pb. Among these techniques, ICP-AES offers fast multi-elemental simultaneous analysis resulting in a wide range of applications including complex and organic matrices with an extended dynamic range and robust matrix tolerance (Sereshti, *et al.*, 2012).

However, in order to enhance sensitivity and selectivity of the method and improve the limit of detection, a separation and/or a preconcentration prior to the analysis are necessary. Therefore, the application of various sample preparation methods including hydride generation (HG) (Soylak and Tuzen, 2008), cloud point extraction (CPE) (Schwingel Ribeiro, *et al.*, 2013; Shah, *et al.*, 2011; Citak and Tuzen, 2010; Candir, *et al.*, 2008), microwave-assisted digestion (MAD) (Burguera, *et al.*, 1999), solid phase extraction (SPE) (Tokman, *et al.*, 2003; Uzun, *et al.*, 2001), liquid-liquid extraction (LLE) (Minagawa, *et al.*, 1993), dispersive liquid–liquid microextraction (DLLME) (Zhou, *et al.*, 2011), hollow fiber-solid phase microextraction (HF-SPE) (Es'haghi, *et al.*, 2011), coprecipitation (Kagaya, *et al.*, 2005) and ultrasound assisted emulsification microextraction (USAEME) (Sereshti, *et al.*, 2012) have been reported for this purpose.

The USAEME method is proposed as an efficient, simple, rapid and non-expensive alternative to other extraction techniques. Besides, it is environmentally friendly because of the low organic solvent consumption and easy to automate (Regueiro, *et al.*, 2008). It is based on the emulsiûcation of a micro-volume of organic extraction solvent in an aqueous sample by ultrasound and further separation of both liquid phases by centrifugation. The application of ultrasound accelerates the mass-transfer process between two immiscible phases, which together with the large surface of contact between both phases leads

to an increment in the extraction efficiency at a minimum time (Regueiro, *et al.*, 2009). The USAEME method has been applied to the determination of heavy metals in different samples such as tea (Khayatian and Hassanpoor, 2012), Oscillatoria and Juncus littoralis plants (Sereshti, *et al.*, 2011), urine (Sereshti, *et al.*, 2012), and water samples (Sereshti, *et al.*, 2012; Karimi, *et al.*, 2012).

In the present work, the capability of Azolla was studied for accumulation of bismuth (Bi), indium (In) and lead (Pb).

This study is to complete the previous studies concerning the determination of heavy metal uptake by Azolla. Therefore, a simple, fast, sensitive and reliable analytical method was developed for trace analysis of heavy metals such as Bi, In and Pb as model analytes in *Azolla filiculoides* Lam. The USAEME method coupled with ICP-AES was applied for this purpose. Calix [4] pyrrole was used as a chelating agent for extraction of the analytes from aqueous sample solution. The extraction process was optimized by using a central composite design.

MATERIALS & METHODS

A Vista-MPX ICP-AES (Varian Inc., Melbourne, Victoria 3170, Australia) equipped with a slurry nebulizer and a charge coupled device detector was used for simultaneous determination of the analytes (Pb, Bi and In). The atomic emission of each element was measured at 220.353 nm for Bi, 325.609 nm for In and 223.061 nm for Pb. An Eurosonic 4D (Euronda, Montecchio Precalcino (Vincenza) Italy) ultrasonic water bath with a temperature control and a digital timer was used to assist the emulsification of the organic extraction solvent in aqueous sample solutions. A Metrohm 691 pH meter (CH-9100 Herisau, Switzerland) with a combined glass electrode was used for the pH measurements. Centrifuges were performed by using a Hermel-Z 200A (Wehingen-Germany). A 250 μL Hamilton syringe (Bonaduz, Switzerland) was used to inject organic extraction solvent into the aqueous sample solutions.

Chloroform, carbon tetrachloride, tetrachloroethylene, chlorobenzene, ethanol, acetone, *p*-toluene sulfonic acid and sodium chloride with the purity higher than 99%, HNO_3 (65%, extra pure), potassium dihydrogen phosphate and sodium hydroxide were purchased from Merck Chemicals (Darmstadt, Germany). The plant Azolla *filiculoides* Lam. was collected from Anzali wetland (Anzali, Guilan province, north of Iran) and dried under shade at room temperature for 72 h.

Calix [4] pyrrole (the chelating agent) was synthesized by acid catalyzed condensation of pyrrole

and acetone. A mixture of pyrrole (0.5 mL, 7.23 mmol) and acetone (0.7 mL, 9.37 mmol) was dissolved in 12 mL of ethanol. Then, *p*-toluene sulfonic acid (0.9 mL, 0.171 mmol) was added to the reaction mixture while it was stirred. The resulting mixture was allowed to stir overnight at room temperature. Afterward, the reaction was stopped and the mixture was filtered on a buchner funnel and the filtrate was washed with several small portions of ethanol. Finally, the product was collected and dried in air at room temperature [0.74 g (96%)].

Designing the experiments for central composite design, analyzing and modeling the data, analysis of variance and constructing the related plots were performed by using the trial version of "Design-Expert 7.1.3" (Stat-Ease Inc., Minneapolis, USA).

Procedure. Firstly, 10 mL of a sample solution containing 200 µg/L of Pb, Bi and In with the pH value of 7.5 (buffer solution of potassium dihydrogen phosphate/sodium hydroxide) was placed in a 12 mL conical glass test tube. Then, 1 mL of an aqueous solution of NaCl 6.75 % (w/v) and 1 mL of ethanol/water solution of calix [4] pyrrole with the concentration of 1200 mg/L were added to it. Therewith, Bi (III), In (III) and Pb (II) react with calix [4] pyrrole to form the related chelates (Fig. 1). Then, the tube was immersed into the ultrasonic water bath and 130 µL of tetrachloroethylene (extraction solvent) was injected slowly into the sample solution by using a 250 µL syringe. Thereby, a cloudy solution was formed under ultrasonication for 4 min at room temperature. In this step, the chelated analytes with calix [4] pyrrole are extracted into the organic phase. Then, the resulted emulsion was disrupted by centrifugation at 4000 rpm for 3 min. The sedimented organic phase was removed by a microsyringe and dried at 90 ˚C in an oven. Finally, 1 mL of HNO_3 (1 mol L^{-1}) was added to the residue and the resulting solution was analyzed by ICP-AES.

RESULTS & DISCUSSION

The extraction solvent is critical for developing an efficient USAEME technique since its physicochemical properties govern the emulsification phenomenon, and consequently, the extraction efficiency (Fontana, *et al*., 2009). The characteristics such as higher density than water, low solubility in water, high extraction capability for the chelated analytes and stable emulsion in the presence of ultrasound was considered for the extraction solvent. Therefore, tetrachloroethylene (C_2Cl_4, density: 1.62 g/mL), chloroform ($CHCl_3$, density: 1.48 g/mL), carbon tetrachloride (CCl_4, density: 1.59 g/mL) and chlorobenzene (C_6H_5Cl, density: 1.11 g/mL) were examined for this purpose. The compatibility of these solvents with the microextraction method was investigated by injection of 100 µL of each solvent into 10 mL of the solutions containing 200 µg/L of the analytes (Bi, In and Pb). Among these solvents, the highest percent recoveries (Bi, 89%; In, 91% and Pb, 86%) were achieved by using tetrachloroethylene (Fig. 2). Therefore, it was selected as the extraction solvent in the following experiments.

In order to find the best extraction conditions for the analytes the effective parameters (factors) of USAEME method including volume of extraction solvent (*E*), sonication time (*t*), salt concentration (*S*), concentration of chelating agent (*L*) and pH (*P*) were optimized using a half-fraction factorial central composite design (CCD) (Morgan, 1991). The design was consisted of 2^{f-1} factorial points, $2f$ axial points, and N_0 center points, where *f* is the number of factors. At the central point, all factors are set at their midpoint, and it is usually repeated to get a good estimate of experimental error. The axial points are located at +á and –á from the center of the experimental domain. The value of á needed to ensure rotatability was calculated equal to ±2 by using Eq. (1).

$$\alpha = \sqrt[4]{2^{f-1}} \qquad (1)$$

Table 1 shows the factors, their symbols and levels including axial points (±α), factorial points (±1) and central points (0).
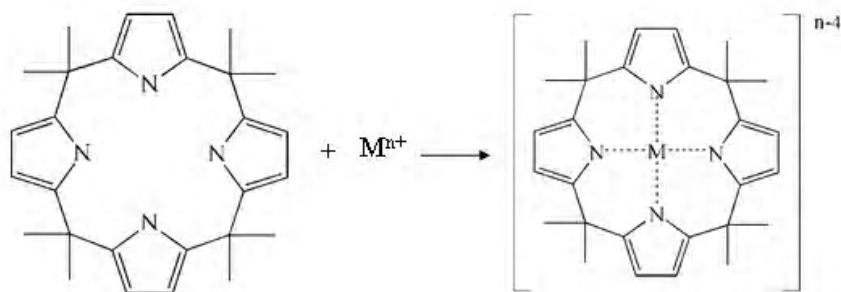

Fig. 1. The schematic complexation of calix [4] pyrrole and the analytes (M^{n+}: Bi^{3+}, In^{3+} and Pb^{2+})
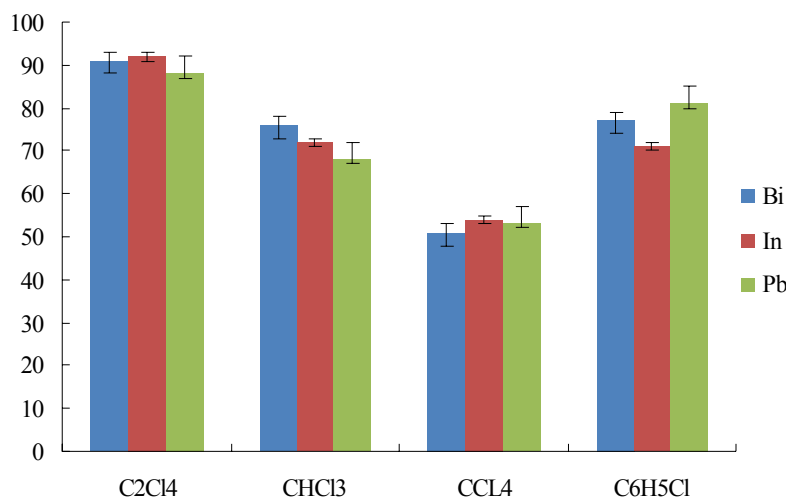
Fig. 2. Effect of various extraction solvent on the extraction recoveries of Bi³⁺, In³⁺ and Pb²⁺

Table 1. Independent factors, their symbols and levels of the central composite design

Factor	Symbol	Levels				
		-α	-1	0	+1	+α
Volume of extraction solvent (μL)	E	80	110	140	170	200
Sonication time (min)	T	1	3	5	7	9
Salt concentration (w/v %)	S	0	2.5	5	7.5	10
Concentration of chelating agent (mg/L)	L	300	600	900	1200	1500
pH	P	2	4	6	8	10

The total number of experiments (*N*) needed to perform the design was obtained from Eq. (2) equal to 32.

$$N = 2^{f-1} + 2f + N_0 \qquad (2)$$

To reduce the effect of uncontrolled factors, the order of the experiments was randomized. After performing the design and recording the results, a quadratic response surface model with the most reasonable statistics fitted to the experimental data was obtained and shown in Eq. (3). This model consisted of five main effects (*L, E, T, P* and *S*), six two-factor interactions (*LT, LS, ET, ES, TS* and *PS*), and four curvature effects (L^2, E^2, T^2, P^2 and S^2):

$$Y = b_0 + b_1 L + b_2 E + b_3 T + b_4 P + b_5 S + b_6 LT + b_7 LS + b_8 ET + b_9 ES + b_{10} TS + b_{11} PS + b_{12} L^2 + b_{13} E^2 + b_{14} T^2 + b_{15} P^2 + b_{16} S^2 \qquad (3)$$

$b_0 = 98.6$; $b_1 = -5.0$; $b_2 = -4.2$; $b_3 = -1.8$; $b_4 = 21.7$; $b_5 = 4.4$; $b_6 = -3.9$; $b_7 = 3.7$; $b_8 = 3.2$; $b_9 = 4.7$; $b_{10} = -3.8$; $b_{11} = 5.4$; $b_{12} = 4.3$; $b_{13} = -11.3$; $b_{14} = -7.9$; $b_{15} = -11.5$; $b_{16} = -8.7$.

where Y is the response (average extraction recovery) b_0 is the intercept, and b_1 to b_{16} are the coefficients. The sign of the coefficients of the model terms (positive or negative) defines the direction of the relationship between the related effect and the response. The positive sign indicates that as the value of one effect changes, the value of the response changes in the same direction too, while for the negative sign the response operates in the opposite direction.

To evaluate the precision, fitness and significance of the model, and the effect of individual factors and their interactions on the response the analysis of variance (ANOVA) was performed (Table 2). The *F-value* which is the test for comparing the variance associated with a term with the residual variance, implies that the model is significant. The *lack of fit* that is the weighted sum of squared deviations between the mean response at each factor level and the corresponding fitted value, with the *F-value* of 1.26 and *p-value* (probability of error value) of 0.41 is not significant for the model. The model terms with *p-values* less than 0.05 are signiûcant for 95% conûdence intervals and values greater than 0.1000 indicate the model terms are not significant. In this case *L, E, P, S, ES, PS, L², E², T², P²* and *S²* are the significant model terms.

Table 2. Analysis of variance (ANOVA) for the central composite design

Source[a]	Sum of squares[b]	d.f.[c]	Mean square[d]	F-value[e]	p-value, prob>F[f]	Significance
Model	25475.16	20	1273.76	20.5	< 0.0001	significant
L	607.02	1	607.02	9.77	0.0097	significant
E	422.52	1	422.52	6.80	0.0244	significant
T	74.55	1	74.55	1.20	0.2968	insignificant
P	11357.85	1	11357.85	182.77	< 0.0001	significant
S	462.00	1	462	7.43	0.0197	significant
LE	27.83	1	27.83	0.45	0.5172	insignificant
LT	245.71	1	245.71	3.95	0.0722	
LP	21.86	1	21.86	0.35	0.5651	insignificant
LS	219.78	1	219.78	3.54	0.0867	
ET	164.48	1	164.48	2.65	0.1320	insignificant
EP	3.52	1	3.52	0.057	0.8164	insignificant
ES	354.38	1	354.38	5.70	0.0360	significant
TP	0.68	1	0.68	0.01	0.9185	insignificant
TS	234.86	1	234.86	3.78	0.0779	
PS	465.48	1	465.48	7.49	0.0193	significant
L^2	536.94	1	536.94	8.64	0.0135	significant
E^2	3743.31	1	3743.31	60.24	< 0.0001	significant
T^2	1811.78	1	1811.78	29.15	0.0002	significant
P^2	3868.61	1	3868.61	62.25	< 0.0001	significant
S^2	2199.41	1	2199.41	35.39	< 0.0001	significant
Residual[g]	683.58	11	62.14			
Lack of fit[h]	410.83	6	68.47	1.26	0.4106	insignificant
Pure error[i]	272.75	5	54.55			
Cor. Total[j]	26158.74	31				

[a]Source of variation; [b]Sum of the squared differences between the average values and the overall mean; [c]Degrees of freedom; [d]Sum of squares divided by d.f.; [e]Test for comparing term variance with residual (error) variance; [f]Probability of seeing the observed F-value if the null hypothesis is true; [g]Consists of terms used to estimate the experimental error; [h]Variation of the data around the fitted model; [i]Variation in the response in replicated design points; [j]Totals of all information corrected for the mean.

The elimination of the insigniûcant terms such as *LE, LP, EP* and *TP* improved the regression model and simplified the equation. Nevertheless, in this case the removing terms of C, BC and AE resulted in a reduced predicted R^2 and the model precision thus cannot be removed. The quality of fit of the polynomial model equation was expressed by the coefficients of determination (R^2, adjusted-R^2 and "adequate precision"). R^2 is a measure of the amount of variation around the mean explained by the model and is equal to 0.9718. The adjusted-R^2 is a measure of the amount of variation around the mean explained by the model, adjusted for the number of terms in the model. The adjusted-R^2 decreases as the number of terms in the model increases if those additional terms don not add value to the model. Here, adjusted-R^2 is equal to 0.9417. Adequate precision is a signal-to-noise ratio. It compares the range of the predicted values at the design points to the average prediction error (Eq. (4)). Ratios greater than 4 indicate adequate model discrimination. In this case, the adequate precision is equal to 22.82.

(4)

$$\left[\frac{\max(\hat{Y}) - \min(\hat{Y})}{\sqrt{\overline{V}(\hat{Y})}}\right] > 4, \overline{V}(\hat{Y}) = \frac{1}{n}\sum_{i=1}^{n} V(\hat{Y}) = \frac{p\sigma^2}{n}$$

Ý is the predicted value, *p* is the number of model parameters (including the intercept (b_0) and any block coefficients), σ^2=residual MS from ANOVA table, and *n* is the number of experiments.

In order to study the quality of influence of the significant interaction effect of the variables on the response, three-dimensional (3D) response surface and contour plots were constructed. These plots represent the relationship between the response and levels of two factors simultaneously, while the other factors are ûxed at their central levels. Fig. 3 shows the response surface and contour plots of the effects of the extraction solvent volume and salt concentration on the response. Both factors demonstrated quadratic effects on the extraction efficiency. Therefore, the response increased up to about 130 μL of extraction solvent followed by a decline with its further increase. Salt concentration also displayed a quadratic effect on the response yielding the maximum between 5.0 and 6.0 percent. The effect of pH and salt concentration on the response is presented in Fig. 4. The response increased with increasing the pH values and obtained its maximum at pH of about 7.5. Salt concentration also displayed a quadratic effect on the response yielding the maximum between 5.5 and 6.5 percent.

Finally, the optimal conditions were calculated based on the fitted model and the desirability function. It reflects the desirable ranges for each response (d_i). The desirable ranges are from zero to one (least to most desirable respectively). The simultaneous objective function is a geometric mean of all transformed responses:

$$(5)$$

$$D = (d_1 \times d_2 \times ... \times d_n)^{\frac{1}{n}} = (\prod_{i=1}^{n} d_i)^{\frac{1}{n}}$$

where *n* is the number of responses in the measure. If any of the responses or factors fall outside their desirability range, the overall function becomes zero. Therefore, the optimum set points were: 130 μL for volume of extraction solvent, 4 min for ultrasonic time, 6.75 % (w/v) for salt concentration, 890 mg/L for concentration of ligand and 7.5 for pH.

Under the optimal conditions, the analytical figures of merit such as linear dynamic range (LDR), limit of detection (LOD), determination coefficients (R^2), relative standard deviations (RSD) and extraction recovery (ER) were determined. The calibration curves were constructed with twelve concentration levels in the range of 1–1500 μg/L. The calibration data were summarized in Table 3. The limits of detection (LODs) based on $3S_d/m$ (where S_d is the standard deviation of the blank and *m* is the slope of calibration graph) were 0.76, 0.57 and 0.84 for Bi, In and Pb measurements. The

relative standard deviations (C=200 μg/L, n=7) were, 2.4% for Bi, 3.7% for In and 5.1% for Pb. Extraction recoveries were 98% for Bi, 97% for In and 93% for Pb. The preconcentration factor (PF) was calculated equal to 12 based on the following equation:

$$PF = \frac{C_{sedimented}}{C_0} \qquad (6)$$

where *PF*, $C_{sedimented}$ and C_0 are the preconcentration factor, analyte concentration in the sedimented phase and initial analyte concentration in the sample, respectively.

In order to evaluate the selectivity of the method for the determination of trace levels of the analytes (Bi^{3+}, In^{3+} and Pb^{2+}), the potential interference from coexisting common ions on the recovery of analytes was investigated. For this purpose, the coexisting ions with various ratios to the analytes were added one by one to the solutions containing 200 μg/L of the analytes and then treated according to the proposed procedure (section 2.5). The results in Table 4 show that percent recoveries of the analytes even in the presence of high concentrations of other coexisting ions are higher than 90%.

In order to study the accuracy and applicability of the proposed method, the determination of Bi, In Pb in two certified reference materials (NIST- 1515 Apple leaves and IAEA-392 Algae material) was carried out. The results presented in Table 5 are in good agreement with the certified values.

To evaluate the applicability of the validated method on real samples, determination of the analytes (Bi^{3+}, In^{3+} and Pb^{2+}) in the plant *Azolla filiculoides* Lam. was investigated using optimized USAEME-ICP-AES. For this purpose, 1 g of the dried, powdered and homogenized plant materials was placed in a 50 mL beaker, and then 5 mL HNO_3 and 2 mL $HClO_4$ was added to it and heated to 150 °C for 1 h under a fume hood. After digestion of the plant materials, the solution was filtered to separate the fine solid particles of the plant from the solution. Then, the solution was diluted to 50 mL with double distilled water and was treated according to the procedure (section 2.5) and finally analyzed by ICP-AES. The results are presented in Table 6.

A comparison of the proposed method with other previously reported studies based on some analytical characteristics (LDR, LOD and RSD) is given in Table 7. The linear dynamic range of the presented method is considerably extensive than that of the other methods. The precision of the method based on RSD is better than that of the others. Furthermore, the LODs achieved with this method for Bi, In and Pb is better than those
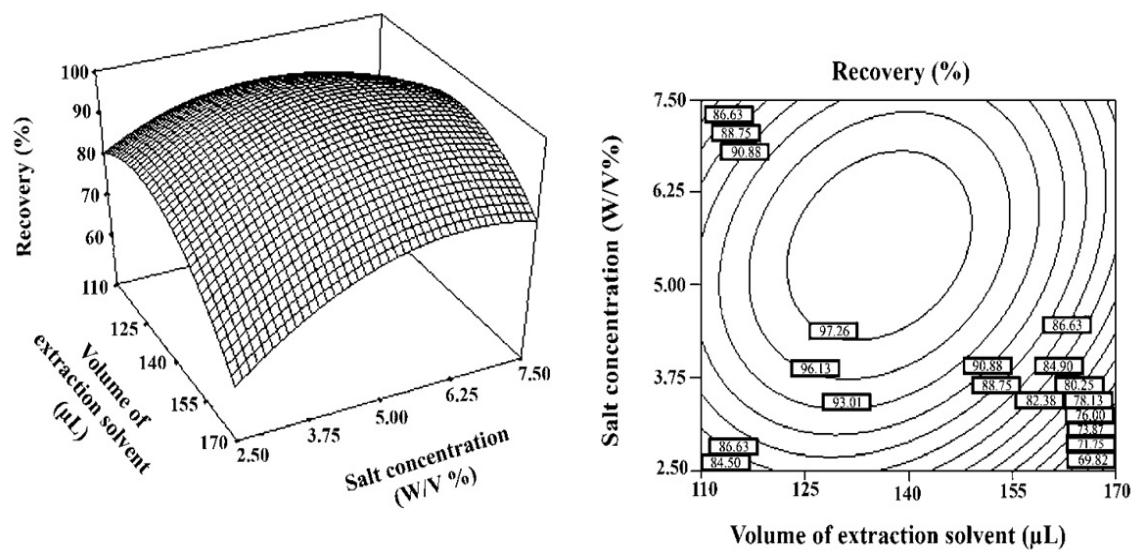
Fig. 3. Response surface and contour plots for the effect of extraction solvent volume and salt concentration on the average extraction recovery. Conditions: concentration of ligand, 900 mg/L; sonication time, 5 min; and pH, 6
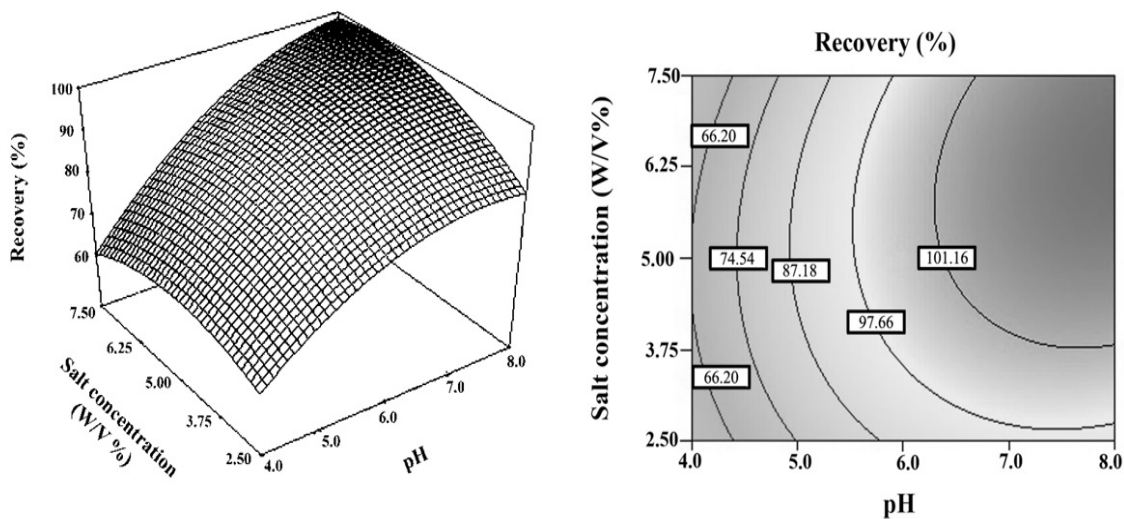

Fig. 4. Response surface and contour plots for the effect of salt concentration and pH on the average extraction recovery. Conditions: extraction solvent volume, 140 µL; concentration of ligand, 900 mg/L; sonication time, 5 min

Table 3.The calibration data for determination of Bi^{3+}, In^{3+} and Pb^{2+}

Metal ions	Calibration equation	Concentration levels[a]	R^2
Bi^{3+}	$I^{[b]} = 0.5068C^{[c]} + 3.5266$	12	0.9983
In^{3+}	$I = 1.0127C - 7.7371$	12	0.9974
Pb^{2+}	$I = 1.3539C + 1.9802$	12	0.9997

[a]Three replicates for each level; [b]I , intensity (counts/s); [c]C, concentration (mg/L)

Table 4. Effect of common coexisting ions on the recovery of Pb (II), In (III) and Bi (III)

Recovery (%)			Ratio of coexisting ions to the analytes (w/w) [†]	Coexisting ion
Pb^{2+}	Bi^{3+}	In^{3+}		
95	97	103	500	K^+
96	98	96	200	Ca^{2+}
105	102	98	50	Cr^{3+}
102	103	99	20	Fe^{3+}
98	97	95	50	Ni^{2+}
93	97	103	100	Cu^{2+}
104	93	94	20	Ag^+
101	97	98	100	Al^{3+}
101	102	93	50	Zn^{2+}
98	96	103	100	CO_3^{2-}
102	99	97	100	SO_4^{2-}
104	94	95	20	$Cr_2O_7^{2-}$

[†] The concentration of analytes was 200 µg/L

Table 5. Determination of Bi, In and Pb in two certified reference materials

Sample	Bi^{3+}	In^{3+}	Pb^{3+}
NIST- 1515 Apple leaves			
Certified value±S.D.[a] (ng/g)	n.r. [b]	n.r.	470±20
Added (ng/g)	100	100	100
Amount found±S.D. (ng/g)	101±2	97±1	572±6
Relative Recovery (%)	101	97	102
IAEA-392 Algae material			
Certified value±S.D. (ng/g)	n.r.	n.r.	574±19
Added (ng/g)	100	100	100
Amount found±S.D. (ng/g)	98±4	97±3	675±8
Relative Recovery (%)	98	97	101

[a] Average±Standard deviation (n =3).
[b] Not reported.

Table 6. Determination of Pb, Bi and In in *Azolla filiculoides* Lam

Sample	Pb^{2+}	Bi^{3+}	In^{3+}
Azolla ± S.D.[a] (ng/g)	116±6	74±3	55±2
Added (ng/g)	100	100	100
Found ± S.D. (ng/g)	212±4	168±4	152±3
Relative Recovery (%)	96	94	97

[a] Standard deviation (n =3)

Table 7. Comparison of the proposed method with other methods

Element	Method	Analysis	LDR (µg/L)	LOD (µg/L)	RSD %	Ref.
Pb,Bi,In	USAEME	ICP-AES	1-1000,1-1500,1-1500	0.84,0.57,0.76	5.1,2.4,3.7	This work
Pb,Bi,In	CM[a]	GFAAS	5-80,20-400,20-400	1.3,8.5,4	-	(Sutton, *et al.*, 1997)
Pb,Bi	LL[b]-CPE	FAAS	0.5-12,0.5-6.5	7.2,0.8	-	(Sun and Wu, 2011)
Pb,Bi	Coprecipitation	ICP-AES	0.5-25	0.137,0.073	-	(Yilmaz, et al., 2012)
Pb,Bi	SPE	GF-AAS	-	0.5, 0.5	2	(Minagawa, *et al.*, 1993)
Pb,Bi	SPE	FAAS	-	19,19	< 9	(Es'haghi, *et al.*, 2011)

[a]Chemical modification; [b]Ligandless-cloud point extraction.

of LL-CPE-FAAS, SPE-FAAS and CM-GF-AAS methods and comparable with SPE-GF-AAS and coprecipitation-ICP-AES methods.

CONCLUSION

In this study, the combination of an optimized-USAEME method and ICP-AES was successfully applied for the extraction and determination of Bi, In and Pb in the plant *Azolla filiculoides* Lam. Calix [4] pyrrole was used as a selective complexing agent for the extraction of the analytes. Ultrasound at room temperature was used to enhance the extraction efficiency. A central composite design with the least number of experiments was applied for optimization of the effective parameters of the method and modeling the data. The experimental figures of merit and statistical analysis of the data demonstrated validity of the results. A relatively wide linear dynamic range, satisfactory precision and detection limit are the advantages of the method. In addition, the proposed method is simple, fast, inexpensive and environmentally friendly with consuming low volume (micro-liter) of organic solvent and low amounts of plant materials.

REFERENCES

Acar, O., Türker, A. R. and Klllç, Z. (2000). Determination of bismuth, indium and lead in spiked sea water by electrothermal atomic absorption spectrometry using tungsten containing chemical modifiers. Spectrochim. Acta B, **55,** 1635–1641.

Burguera, J.L., Burguera, M., Rivas, C., Rondon, C., Carrero, P. and Gallignani, M. (1999). Determination of bismuth in biological samples using on-line flow-injection microwave-assisted mineralization and precipitation:dissolution for electrothermal atomic absorption spectrometry. Talanta, **48,** 885–893.

Candir, S., Narin, I. and Soylak, M. (2008). Ligandless cloud point extraction of Cr(III), Pb(II), Cu(II), Ni(II), Bi(III), and Cd(II) ions in environmental samples with Tween 80 and flame atomic absorption spectrometric determination. Talanta, **77,** 289–293.

Citak, D. and Tuzen M. (2010). A novel preconcentration procedure using cloud point extraction for determination of lead, cobalt and copper in water and food samples using flame atomic absorption spectrometry. Food Chem. Toxicol., **48,** 1399–1404.

Es'haghi, Z., Khalili, M., Khazaeifar, A. and Rounaghi, G. H. (2011). Simultaneous extraction and determination of lead, cadmium and copper in rice samples by a new pre-concentration technique: Hollow fiber solid phase microextraction combined with differential pulse anodic stripping voltammetry. Electrochim. Acta, **56,** 3139–3146.

Fontana, A., Wuilloud, R., Martinez, L. and Altamirano, J. (2009). Simple approach based on ultrasound-assisted emulsification-microextraction for determination of polibrominated flame retardants in water samples by gas chromatography–mass spectrometry. J. Chromatogr. A, **1216,** 147–153.

Fu, J., Zhou, Q., Liu, J., Liu, W., Wang, T., Zhang, Q. and Jiang, G. (2008). High levels of heavy metals in rice (Oryza sativa L.) from a typical E-waste recycling area in southeast China and its potential risk to human health. Chemosphere, **71,** 1269–1275.

Ganji, M. T., Khosravi, M., Rakhshaee, R. (2005). Biosorption of Pb, Cd, Cu and Zn from the wastewater by treated *Azolla filiculoides* with $H_2O_2/MgCl_2$. Int. J. Environ. Sci. Tech., **1,** 265–271.

Ghorbanzadeh Mashkani, S. and Tajer Mohammad Ghazvini, P. (2009). Biotechnological potential of Azolla filiculoides for biosorption of Cs and Sr: Application of micro-PIXE for measurement of biosorption. Biores. Tech., **100,** 1915–1921.

Gumus, G., Filik, H. and Demirata, B. (2005). Determination of bismuth and zinc in pharmaceuticals by first derivative UV–Visible spectrophotometry. Anal. Chim. Acta, **547,** 138–143.

Kagaya, S., Araki, Y., Hirai, N. and Hasegawa, K. (2005). Coprecipitation with yttrium phosphate as a separation technique for iron(III), lead, and bismuth from cobalt, nickel, and copper matrices. Talanta, **67,** 90–97.

Karimi, M., Sereshti, H. and Samadi, S. (2012). Ligandless-ultrasound assisted emulsification microextraction followed by inductively coupled plasma-optical emission spectrometry for simultaneous determination of heavy metals in water samplesIntern. J. Environ. Anal. Chem., **1,** 1–15.

Khayatian, G. and Hassanpoor, S. (2012). Ultrasound assisted emulsification microextraction based on dimetyl (*E*)-2-[(*Z*)-1-acetyl)-2-hydroxy-1-propenyl]-2-butenedioate for determination of total amount of Iron in water and tea samples. J. Chin. Chem. Soc., **59,** 659–666.

Khosravi, M., Rakhshaee, R., Ganji, M. T. (2005). Pre-treatment processes of *Azolla filiculoides* to remove Pb(II), Cd(II), Ni(II) and Zn(II) from aqueous solution in the batch and fixed-bed reactors. J. Hazard. Mater., **127,** 228–237.

Kokkinos, C., Economou, A. and Raptis, I. (2012). Microfabricated disposable lab-on-a-chip sensors with integrated bismuth microelectrode arrays for voltammetric determination of trace metals. Anal. Chim. Acta, **710,** 1–8.

Liu, R., Wu, P., Xu, K., Lv, Y. and Hou, X. (2008). Highly sensitive and interference-free determination of bismuth in environmental samples by electrothermal vaporization atomic fluorescence spectrometry after hydride trapping on iridium-coated tungsten coil Spectrochim. Acta B, **63,** 704–709.

Minagawa, A., Sawada, K. and Suzuki, T. (1993). Determination of bismuth(III) by graphite furnace atomic absorption spectrometry combined with liquid-liquid extraction with trioctylmethylammonium nitrate. Anal. Chim. Acta, **278,** 287–292.

Morgan, E. (1991). Chemometrics: Experimental design. (London: Basic Books).

Rakhshaee, R., Khosravi, M., Ganji, M. T. (2006). Kinetic modeling and thermodynamic study to remove Pb(II), Cd(II), Ni(II) and Zn(II) from aqueous solution using dead and living *Azolla filiculoides*. J. Hazard. Mater., **134,** 120–129.

Rao, P. V. C. and Koshy, V. J. (1993). Voltammetric determination of indium in aluminia supported catalysts. Talanta, **40,** 761–764.

Regueiro, J., Llompart, M., Garcia-Jares, C., Garcia-Monteagudo, J. C. and Cela, R. (2008). Ultrasound-assisted emulsification–microextraction of emergent contaminants and pesticides in environmental waters. J. Chromatogr. A, **1190,** 27–38.

Regueiro, J., Llompart, M., Psillakis, E., Garcia-Monteagudo, J. C. and Garcia-Jares, C. (2009). Ultrasound-assisted emulsification–microextraction of phenolic preservatives in water. Talanta, **79,** 1387–1397.

Sánchez-Viveros, G., González-Mendoza, D., Alarcón, A. and Ferrera-Cerrato, R. (2010). Copper effects on photosynthetic activity and membrane leakage of *Azolla filiculoides* and *A. caroliniana*. Int. J. Agric. Biol., **12,** 365–369.

Sastre, J., Sahuquillo, A., Vidal, M. and Rauret, G. (2002). Determination of Cd, Cu, Pb and Zn in environmental samples: microwave-assisted total digestion versus aqua regia and nitric acid extraction. Anal. Chim. Acta, **462,** 59–72.

Schwingel Ribeiro, A., Zezzi Arruda, M.A. and Cadore, S. (2002). Determination of bismuth in metallurgical materials using a quartz tube atomizer with tungsten coil and flow injection-hydridegeneration atomic absorption spectrometry. Spectrochim. Acta B, **57,** 2113–2120.

Sereshti, H., Bakhtiari, S. and Khojeh, V. (2011). Simultaneous determination of aluminum, copper, iron and zinc in Oscillatoria, Juncus littoralis tissues and wetland water samples by ultrasound-assisted emulsification-microextraction combined with ICP-OES. Anal. Methods, **3,** 2936–2943.

Sereshti, H., Entezari-Heravi, Y. and Samadi, S. (2012). Optimized ultrasound-assisted emulsification microextraction for simultaneous trace multielement determination of heavy metals in real water samples by ICP-OES. Talanta, **97,** 235–241.

Sereshti, H., Khojeh, V., Karimi, M. and Samadi, S. (2012). Ligandless-ultrasound-assisted emulsification-microextraction combined with inductively coupled plasma-optical emission spectrometry for simultaneous determination of heavy metals in water samples. Anal. Methods, **4,** 236–242.

Sereshti, H., Rohani-Far, A. and Samadi, S. (2012). Optimized ultrasound-assisted emulsification-microextraction followed by ICP-OES for simultaneous determination of lanthanum and cerium in urine and water samples. Anal. Lett., **45,** 1426–1439.

Shah, F., Kazi, T. G., Afridi, H. I., Naeemullah, M. B. and Baig, J. A. (2011). Cloud point extraction for determination of lead in blood samples of children, using different ligands prior to analysis by flame atomic absorption spectrometry: A multivariate study. J. Hazard. Mater., **192,** 1132–1139.

Soylak, M. and Tuzen, M. (2008). Coprecipitation of gold(III), palladium(II) and lead(II) for their flame atomic absorption spectrometric determinations. J. Hazard. Mater., **152,** 656–661.

Sun, M. and Wu, Q. (2011). Determination of trace bismuth in human serum by cloud point extraction coupled flow injection inductively coupled plasma optical emission spectrometry. J. Hazard. Mater., **192,** 935–939.

Sutton, R. M. C., Hill, S. J. and Jones, P. (1997). High-performance chelation ion chromatography for the determination of traces of bismuth in lead by means of a novel hypercrosslinked polystyrene resin. J. Chromatogr. A, **789,** 389–394.

Teixeira, M. F. S. and Fatibello-Filho, O. (2001). Flow injection potentiometric determination of bismuth(III) in anti-acid formulationsInt. J. Pharm., **221,** 115–121.

Timofeeva, D. V., Tsapko, Y. V. and Ermakov, S. S. (2011). Stripping coulometry determination of lead and mercury at screen-printed electrodesJ. Electroanal. Chem., **660,** 195–199.

Tokman, N., Akman, S. and Ozcan, M. (2003). Solid-phase extraction of bismuth, lead and nickel from seawater using silica gel modified with 3-aminopropyltriethoxysilane filled in a syringe prior to their determination by graphite furnace atomic absorption spectrometry. Talanta, **59,** 201–205.

Uzun, A., Soylak, M. and Elçi, L. (2001). Preconcentration and separation with Amberlite XAD-4 resin; determination of Cu, Fe, Pb, Ni, Cd and Bi at trace levels in waste water samples by flame atomic absorption spectrometry. Talanta, **54,** 197–202.

Vydra, F. and Vorlíček, J. (1966). Amperometry with two polarisable electrodes chemometric determination of indium. Talanta, **13,** 439–443.

Yasri, N.G., Halabi, A.J., Istamboulie, G. and Noguer, T. (2011). Chronoamperometric determination of lead ions using PEDOT: PSS modified carbon electrodes. Talanta, **85,** 2528–2533.

Yilmaz, V., Arslan, Z. and Rose, L. (2012). Determination of lead by hydride generation inductively coupled plasma mass spectrometry (HG-ICP-MS): On-line generation of plumbane using potassium hexacyanomanganate(III). Anal. Chim. Acta.

Zhang, X., Lin, A.-J., Zhao, F.-J., Xu, G.-Z., Duan, G.-L. and Zhu, Y.-G. (2008). Arsenic accumulation by the aquatic fern Azolla: Comparison of arsenate uptake, speciation and efflux by A. caroliniana and A. filiculoides. Environ. Pollut., **156,** 1149–1155.

Zhao, M., Ducan, J. R., Van Hille, R. P. (1999). Removal and recovery of zinc from solution and electroplating effluent using *Azolla filiculoides*. Wat. Res., **33,** 1516–1522.

Zhou, Q., Zhao, N. and Xie, G. (2011). Determination of lead in environmental waters with dispersive liquid–liquid microextraction prior to atomic fluorescence spectrometry. J. Hazard. Mater., **189,** 48–53.