

Spectrophotometric determination of selected heavy metals in real samples at trace levels by combination of solidified floating organic drop micro-extraction and net analyte signal-based method

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Abstract

Here in a dispersive liquid- liquid microextraction based on solidification of floating organic drop method was developed for simultaneous pre-concentration and trace determination of cadmium, lead and cobalt ions based on their complexes with dithizone and preprocessing spectrophotometric data by net analyte signal based method. The affecting parameters on extraction were investigated and optimized simultaneously. The optimum conditions for pH, extraction solvent, extraction solvent volume, dispersive solvent, dispersive solvent volume, contact time, centrifugation time, solidification time and freezing temperature were 8, 1-ndecanol, 150 microliter, ethanol, 350 microliter, 10 min, 6 min, 5min and 4 °C, respectively. Under these conditions and using a sample containing 15 ml of the cations, the detection limits were $1.1 \mu\text{g L}^{-1}$, $3 \mu\text{g L}^{-1}$ and $5.4 \mu\text{g L}^{-1}$ for cadmium, lead and cobalt, respectively. Because of spectra sever overlapping of complexes throughout studied pH and spectra regions, the net analyte signal ratio spectra method was used to resolve the problem perfectly. Hence, the determination of studied cations can be conducted in the presence of other complexes and unknown interferences. Ultimately, the modified method was successfully applied for determination of aforementioned cations in real samples.

Keywords: Dispersive liquid- liquid microextraction based on solidification of floating organic drop, Simultaneous pre-concentration, Net analyte signal, Cadmium, Lead, Cobalt.

1. Introduction

In recent years, much attention has been focused on determination of heavy metal ions in environmental samples [1-4]. These metals have been extensively used in various industries such as metallurgy, battery,

mining, plating, textile and electronics [5]. Therefore, they are inevitably discharged directly into the environment by rapid industrialization and hence cause pollution the biological and aqueous samples. Some of heavy metals such as cadmium and lead are highly

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detrimental to human beings and animals even in very small amounts [6, 7]. Lead as a poisoning pollutant can severely damage the kidneys, liver, reproduction system, nervous system, gastrointestinal and immune system [8]. Cadmium is highly harmful to lung, kidneys, skin, bones or teeth of creatures [8]. Some of other heavy metals such as cobalt which known as essential elements, if over intake will cause severe toxicological effects [9, 10]. Consequently, determination and monitoring of these elements in different types of aqueous solution is of great interest. Due to trace levels of metals as well as unknown complex matrix in environmental samples along with interferences there is required a preconcentration and extraction step prior to measurement [11-15]. The most important of these methods are: cloud point extraction (CPE) [16], ion exchange (IE) [17], solid-phase micro extraction (SPME) [1, 11-15], ultrasound-assisted dispersive liquid-liquid microextraction [18], and dispersive liquid-liquid microextraction based on solidification of floating organic drop (DLLME-SFO) [19]. Among them, the last due to its some advantages such as high pre-concentration factor, low poisoning using extraction solvent with lower toxicity, simple and fast extraction, rapid phase separation and low consumption of organic solvents was primarily preferred. The widely used spectroscopic methods for determination of enriched analytes include flame atomic absorption spectrometry (FAAS) [11-15] graphite furnace atomic absorption spectrometry (GFAAS) [20], inductively coupled plasma optical emission spectrometry (ICP-OES) [21] and inductively coupled plasma mass spectrometry (ICP-MS) [19]. Although these techniques have the advantages of accuracy, high precision, simultaneous determination, wider dynamic range and low detection limit, however they are much expensive and is not simply available in any laboratory. Therefore, developing a simple and inexpensive spectrophotometry approach for detecting of heavy metals is extremely important.

In this study by combining DLLME-SFO with spectrophotometer instrument equipped with micro

liter cells and by recording the absorbance of extracted dithizone complexes, these metal was successfully determined at trace levels. The spectra of extracted complexes have severe overlap in whole studied spectra regions. Hence, the multivariate calibrations were used for simultaneous determination. In multivariate calibration methods such as classical least squares (CLS) and inverse least squares (ILS) the nature of calibration and prediction set should be identical. In other words, the absorbent components in calibration model should be exist in prediction set. Otherwise, the model will fail to predict the accurate results. In DLLME-SFO measurements, the nature and matrix of environmental samples are unknown. Furthermore, the dithizone as general ligand can extract some metal ions; hence, the calibration samples will be dissimilar to prediction samples. In order to resolve this problem, the net analyte signal (NAS) based calibration method was used so as to preprocessing the spectrophotometric data [22]. NAS is a part of signal which is directly proportional to the model predicted concentration. Mathematically, the NAS is a component of spectra which is orthogonal to space that were built by all spectra except one. In the current work, the combination of NAS and CLS method, which called NAS/CLS, was used to minimize the un-calibrated interferences in calibration set. Moreover, the mean centering ratio spectra technique was utilized to eliminate the interfering effects of extracted ligand by dividing all spectra to ligand spectrum and then by mean centering the resulted spectrum. Here in, the theory of the net analyte signal based methods can be described briefly. The matrices and vectors are used is as follows: an $I \times J$ calibration data matrix M containing responses of I samples at J wavelengths, an $J \times 1$ vector s_x composed of pure spectrum of analyte x at unit concentration and $I \times 1$ calibration concentration c_x vector of interested analyte. The variability of calibration data matrix M is composed of analyte x (M_x) responses and other components responses except analyte (M_{-x})

$$M = M_x + M_{-x} \quad (1)$$

On condition that one analyte of interest exists and provided that linearity is fulfilled, the M_x can be written as follows:

$$M_x = c_x s_x^T \text{ then } M = c_x s_x^T + M_{-x} \quad (2)$$

In calibration context, the net analyte calibration matrix (M_x^*) are vectors which are orthogonal to spaces spanned by the other vectors (M_{-x}) except x [23]. In other words, they are the part of spectra that are orthogonal to the spaces which were created by the spectra of all other analytes except the interested one and is given by the following equation:

$$M_x^* = M R_{NAS} = (M_x + M_{-x}) R_{NAS} = M_x R_{NAS} = c_x s_x^T R_{NAS} \quad (3)$$

Where the R_{NAS} is an $J \times J$ preprocessing matrix which is orthogonal to M_{-x} and removes from calibration matrix all sources of variability except analyte x [24]. Hence:

$$M_x^* = c_x s_x^T R_{NAS} \quad (4)$$

The equation simply written as:

$$M_x^* = c_x (s_x^*)^T \quad (5)$$

Where M_x^* and s_x^* are net analyte calibration spectra matrix and net sensitivity of analyte x , respectively [25]. Thus, simply the net analyte calibration matrix can be fitted to the calibration concentrations (c_x) using classical least squares (CLS) and the calibration coefficient will be obtained as a result of usual least square fit [26]. The aforementioned equation leads to:

$$s_x^* = (M_x^*)^T c_x (c_x^T c_x)^{-1} \quad (6)$$

and this vector will subsequently predict the analyte x concentration in an unknown sample.

The R_{NAS} matrix projects the rows of calibration spectra (M) onto the NAS space [24] and is giving by the following equation:

$$R_{NAS} = I - (M_{-x})^+ M_{-x} \text{ and } M_x^* = M R_{NAS} \text{ then } M_x^* = M [I - (M_{-x})^+ M_{-x}] \quad (7)$$

Where I is an $J \times J$ unitary matrix and $(M_{-x})^+$ is pseudoinverse of M_{-x} . $(M_{-x})^+$ is calculated by applying singular value decomposition using K PCA factors [25] in the following equation:

$$R_{NAS} = I - U U^T \text{ and } M_x^* = M R_{NAS} \text{ then } M_x^* = M [I - U U^T] \quad (8)$$

Where U is an $J \times K$ matrix that contains the K first (with the largest eigenvalues) u_k eigenvectors of squares matrix $[(M_{-x})^+ M_{-x}]$. The optimum number of K can be estimated using cross validation methods [27] or by minimizing the error values in a validation set [24]. In ideal systems, the number of K is equal to the number of components which create the space of M_{-x} . The exact nature of M_{-x} is not well defined in unknown samples. Hence, the numerous and different methods were used to find M_{-x} . However, they all present matrixes whose rows are linear combination of original (M_{-x}) matrix rows and generally these matrixes are also named M_{-x} and they all explain a common aspect [24]. This matrix is calculated by the following equation:

$$M_{-x} = M - c_x s^T \quad (9)$$

Where s is a spectrum or an appropriate linear combination of spectra which is containing the pure spectral profile of analyte x . one of these calculation and even attractive one is the projection of M matrix orthogonal to c_x [28]:

$$M_{-x} = [I - c_x (c_x^T c_x)^{-1} c_x^T] M \quad (10)$$

comparing equation 9 and 10 leads to:

$$s^T = (c_x^T c_x)^{-1} c_x^T M \quad (11)$$

Hence, s^T is a special linear combination of spectra which is containing in matrix M . This unique property makes M_{-x} orthogonal to c_x . This algorithm is named NAP (net analyte preprocessing) [25].

In summary, NAP/CLS is consist of two steps. The first, projection of M orthogonal to c_x in order to find M_{-x} , the space is spanned by all components except analyte x , and the second, projection of M orthogonal to U to get M_x^* , the analyte calibration data, and correlating this matrix to c_x through a CLS procedure (NAP/CLS) [29].

The NAP/CLS in details is as follows:

1 – pre-processing calibration data

1a - projection of M orthogonal to c_x in order to find M_{-x}

$$M_{-x} = [I - c_x (c_x^T c_x)^{-1} c_x^T] M$$

1b - applying SVD on M_{-x} and obtaining U with K factors

1c - projection of M orthogonal to U to get M_x^*

$$M_x^* = M [I - U U^T]$$

2 – pre-processing unknown sample

2a - projection of m orthogonal to U in order to find

$$m_x^*$$

$$m_x^* = [I - U U^T]m$$

where m is an $J \times 1$ vector composed of unknown sample spectrum

3 - classical least square calibration and prediction

3a- CLS calibration to calculate the calibration coefficient s_x^*

$$M_x^* = c_x (s_x^*)^T \rightarrow s_x^* = (M_x^*)^T c_x (c_x^T c_x)^{-1}$$

3b- concentration prediction of x in unknown sample (c_{xun})

$$m_x^* = c_{xun} (s_x^*)^T \rightarrow c_{xun} = (s_x^{*T} s_x^*)^{-1} s_x^{*T} m_x^*$$

It is noted that before NAP all spectra were divided by dithizone spectrum and then mean centered to remove the effects of extracted ligand in extraction.

2. Experimental

2.1. Chemicals and reagents

Hydroxide sodium, ethanol, methanol and acetone with high purity were obtained from Merck (Darmstadt, Germany, www.merck.de). Analytical grade 1-undecanol, acetonitrile, dithizone, $Pb(NO_3)_2$, $Ni(NO_3)_2$, $Cd(NO_3)_2 \cdot 4H_2O$, $Zn(NO_3)_2 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, $Cu(NO_3)_2 \cdot 2.5H_2O$, $Hg(NO_3)_2 \cdot H_2O$, $Fe(NO_3)_3 \cdot 9H_2O$, KNO_3 , $AgNO_3$, NaI , $NaBr$, Na_2CO_3 and Na_2SO_4 were purchased from Sigma- Aldrich company. All the other reagents used were of analytical grade and purchased from Merck (Darmstadt, Germany, www.merck.de). Doubly distilled water was used to preparing, dissolving and diluting the samples.

2.2. Instrumentals and softwares

All absorption spectra were recorded using the double beam Uv-Vis spectrophotometer model UV-1800 (Shimadzu Company) in the wavelength ranges of 350 – 700 nm with one nm increment intervals equipped with 50 microliters quartz cells. All data were saved as ASCII format and were transferred to a personal computer for subsequent manipulation. Written in MATLAB7 2009a (MathWorks Company) software, the homemade m files were used to preprocessing data and mean centering ratio spectra as well as obtaining

net analyte signals at optimization steps. For conducting NAS\CLS calibration as well as obtaining NAS signals in real samples the MULTIVAR program available at www.iquir-conicet.gov.ar/descargas/mvc1.rar were used. The pH measurements were carried out on a digital pH-meter (Metrohm 744) using a combined glass electrode.

2.3. Preparing the samples

1M nitric acid and 1 M sodium hydroxide were employed for pH adjustment. Stock solution of 1000 mg L⁻¹ of cadmium, lead and cobalt were prepared by dissolving the appropriate amount of purchased nitrate standard of their cations in doubly distilled water. Working solutions were prepared daily by suitable dilution. Stock solution of dithizone (1000 ppm) was made daily by dissolving dithizone (Merck, Darmstadt, Germany) in ethanol and was stored in a refrigerator (at 4 degrees Celsius) in brown flask.

2.4. Pretreatment of real water samples

All real water samples were filtered through a 0.45 μ m filter to remove suspended particles then the pH of samples was adjusted to 8 and the proposed procedure was applied.

2.5. DLLME-SFO procedure

An optimized concentration of the ligand (30 microliter of 1000ppm) was added into 15 mL of sample solution containing appropriate amount of cadmium, lead and cobalt ions and the pH of solution was adjusted at 8.0. After that, the mentioned solution was stirred for 10 min under 500 rpm stirring rate to complete formation of cations-ligand complex. Then, 150 microliter of 1-undecanol and 350 microliter ethanol was mixed and injected into aqueous solution rapidly using a syringe and the cloudy solution was appeared quickly. The resulted solution was centrifuged for 6 min at 4500 rpm and was cooled in a refrigerator at 4 degrees Celsius for 5 min. The solidified organic drop (about 100 microliter) at upper phase was collected using a spatula into a vial. The liquid sample in vial at room temperature was transferred to a 50 microliter quartz cell and the absorbance spectrum of extracted analytes was recorded in 350-700 nm ranges with one nm

increment intervals. 15 mL distilled water (without target ions) was extracted under optimized conditions and final extracted solvent (50 μ l) was used as blank solution. Figure 1, provide a scheme for

preconcentration of target ions by the proposed sample preparation method and their trace detection by spectroscopic technique based on net analyte signal.

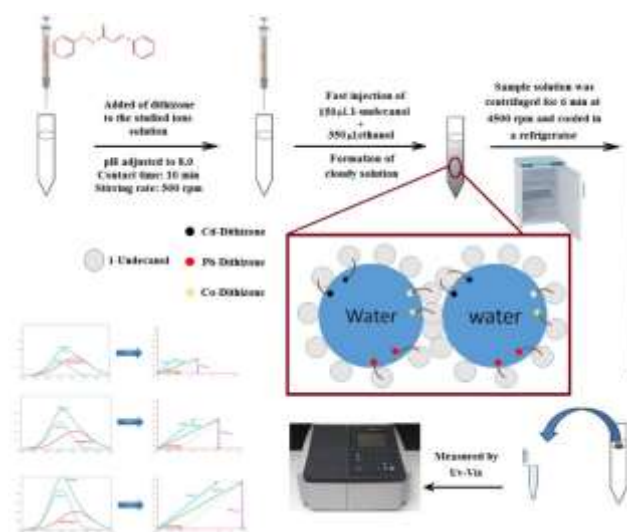


Fig. 1. A scheme for illustration of the proposed method for trace detection of cadmium, lead and cobalt ions.

3. Result and discussion

The performance of the proposed DLLME-SFOD method coupled with spectrophotometric NAS/CLS technique was investigated for determination of cadmium, lead and cobalt at trace level with dithizone as chelating agent. The parameters which affect the extraction and the measurement were studied and optimized. These factors are pH, extraction solvent, extraction solvent volume, dispersive solvent, dispersive solvent volume, maximum wavelength or range of wavelengths, contact time for completing formation of complex, stirring rate, ligand concentration, centrifugation time, solidification time and freezing temperature. For the purpose of optimization, 15 ml doubly distilled water containing the appropriate amount of interest cations was used under various experimental conditions.

3.1. Study on the absorption spectra of complexes in various pH

The pH solution has significant effect on cations extraction with dithizone ligand. Hence, the form of the extracted compounds may be altered by pH variation and subsequently the resulted spectra will be changed. Measuring an analyte with common spectrophotometry

requires a wavelength or a region of wavelengths where other components except analyte lack absorbance signal. Figure 1S, 2S and 3S (Electronic supplementary materials (ESM)) shows the spectra of cadmium, lead and cobalt complexes with dithizone in the pH ranges from 3 to 9, which ranges from 350 nm to 700 nm (in the visible regions). As can be seen, no selective wavelength region for any cations do exist because of severe spectral overlapping of three complexes in whole pH ranges and in whole wavelengths. Therefore, measuring of these cations in the presence of each other and in the presence of other components is impossible using single wavelength calibration methods. As a result, multivariate calibration methods are required to find regression coefficients of analytes spectra with concentration in calibration set and to determine the interested analytes concentrations in unknown samples. In this study, the combined NAP/CLS multivariate calibration method was used to obtain the concentration of cations in all samples. The visible region (350-700 nm) also was examined in recording all spectra.

3.2. pH effect

Formation of cation-dithizone complexes and their extraction into organic solvents are seriously influenced by pH. In acidic solution, as a result of high concentration of protons, protons occupied the active donor sites of dithizone and the dithizone metal complexes cannot be formed effectively. On the other hand, in alkaline medium, the high excess of hydroxide ions will probably precipitate cations as hydroxide form and will decrease the concentration of free cations required to form complex. For this reason, the optimal pH value should be used in the experiments. Because the solubility of studied cation hydroxides are different from each other and also these cations will be determined simultaneously, thus the mixture of three cations were extracted and were investigated in pH ranges between 3 and 9. Then the extracted components were analyzed and the calculated NAS was shown in Figure 4S. As the Figure 4S shows, the pH equal to 8 is considered as optimum value for simultaneous extraction of three studied cations and will be used in further experiments. It is noted that, in all optimization steps the NAS of any complex was recorded as the extraction efficiency.

3.3. The effect of dithizone concentration

In this study, prior to all determinations, all spectra were divided by ligand spectrum and then they were mean centered. Hence, the extracted dithizone has not any interference on determinations. However, the low level of dithizone is causing loss of quantitative extraction of cations. The effect of ligand concentration on extraction efficiency was studied and the results were presented in Figure 5S. The extraction increased by increasing ligand concentration from 0.5 to 2 ppm and then kept constant with subsequent increasing to 2.5 ppm. Hence, 2 ppm ligand concentration was chosen for further studies. In other words, the concentration ratio of dithizone to target ions is approximately equal to 10.

3.4. Effect of contact time

For efficient extraction of target ions, the complete formation of complex between studied ions and

dithizone is vital. For this purpose, the effect of contact time (under stirring rate of 500 rpm) on the extraction efficiency was studied in the range of 5–20 min while the other parameters remained constant and the obtained results were presented in Figure 6S (ESM). The efficiency increased with the increase in time up to 10 min and remained constant thereafter. Consequently, 10 min was selected for next experiments.

3.5. The effect of centrifuging time

Centrifuging is an essential step to condense and float of extracted solvent on the surface of solution and thus centrifuging time has major role on extraction efficiency. The resulted solutions were centrifuged in the range of 2–10 min with 4500 rpm for centrifuging time optimization. The obtained responses indicated that the 6 min is the best choice for this process (Figure 7S).

3.6. Extraction solvent selection

The extraction solvent has significant and considerable role in DLLME-SFOD based systems. The extraction solvent should have special properties required to efficient extraction of analytes are as follows: low solubility in aqueous solution, lower density than water, high efficiency in extraction of analytes, melting point near room temperature and capability of producing floating drop on aqueous solution surface. According to these characteristics, 1-undecanol was selected as solvent extraction.

3.7. The choice of disperser solvent

The disperser solvent should be miscible in both aqueous solution and extraction solvent. Hence solvents such as acetone, acetonitrile, ethanol and methanol were selected and examined in extraction. According to the obtained results in Figure 2, the ethanol is the best disperser solvent and was chosen for further experiments. Ethanol was soluble in 1-undecanol and was miscible with water. According to the obtained data, ethanol enabled to more disperse 1-undecanol as fine particles in aqueous phase and forms a cloudy solution (water/ethanol/1-undecanol) and therefore had higher extraction efficiency. Finally,

ethanol was selected as the disperser solvent due to its low toxicity and higher extraction efficiency.

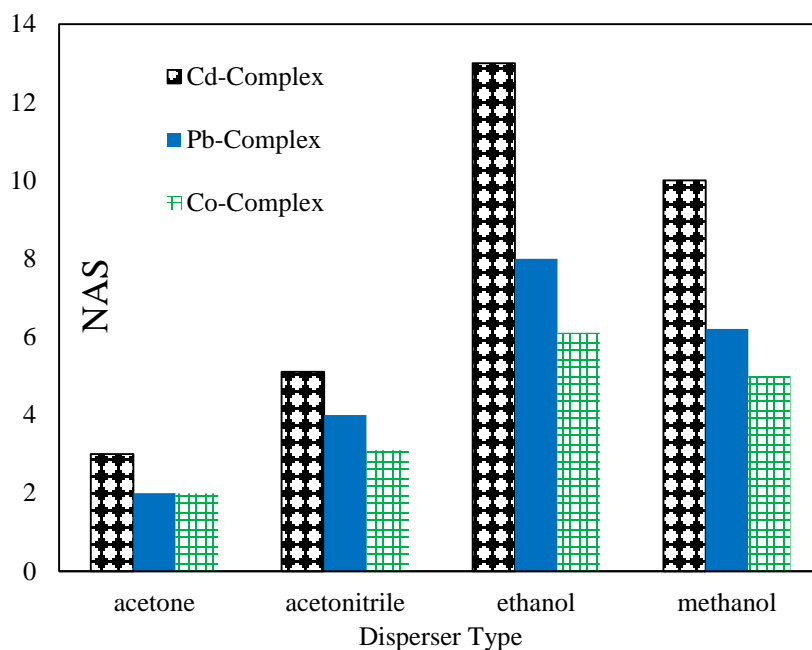


Fig. 2. Type of disperser solvent effect on extraction efficiency (Net Analyte Signal of complexes). Conditions: aqueous volume: 15 ml, Cadmium: 85 ppb, Lead: 85 ppb, Cobalt: 85 ppb, pH: 8, Dithizone: 2ppm, 1-Undecanol: 150 μ L, Disperser: 350 μ L, Contact time: 10 min, Stirring rate: 500 rpm, centrifuging time: 6 min (4500 rpm), Solidification time: 5 min, Freezing temperature: 4 $^{\circ}$ C, Volume of collected extractant: $100 \pm 2\mu$ L.

3.8. Effect of extraction solvent volume

In order to investigate the volume of 1-undecanol on extraction efficiency, the experiments were performed in the presence of various amount of 1-undecanol while the other experimental conditions remained constant (Figure 8S). As can be seen, by increasing 1-undecanol volume up to 150 microliter the extraction increased and thereafter reduced slightly. At first by increasing solvent volume the concentration of analyte increased due to enhance the solvent extraction capacity afterwards the extracted analyte reduce owing to the dilution effect. Then, 150 microliter was chosen as optimum organic solvent volume in the experiments.

3.9. Effect of disperser solvent volume

For the purpose of evaluating the disperser volume on analyte extraction, ethanol volumes of 100 microliter to 500 microliter were applied in the experiments and the resulted signals were displayed in Figure 9S. In lower volumes, the cloudy state in the solution is not formed completely and thus analytes is not extracted

well. On the other hand, in volumes higher than 350 microliter the interested analytes is dissolved in aqueous phase and thus the extraction efficiencies decreases. Hence, 350 microliter of ethanol was considered as optimum value of disperser solvent. It should be noted that the plot of the measured spectrophotometric spectra of extracted dithizone versus pH was presented in Electronic Supplementary Material (Figure 10S).

3.10. Solidification time and freezing temperature

Whereas the 1-undecanol melting point is higher than 10 $^{\circ}$ C, thus the freezing temperature 4 $^{\circ}$ C in 5 minutes is sufficient to solidify it. Consequently, in all experiments the extracted solvent was refrigerated at 4 $^{\circ}$ C in 5 minutes.

3.11. Effect of diverse ions

Although NAP/CLS method have capability of determination of analyte in the presence of other components, the efficiency of the proposed method in the preconcentration and determination of cadmium,

lead and cobalt was evaluated in the presence of common existing anions and cations in aqueous solution. For this purpose, diverse anions and cations with concentration ratio of 100 relative to analytes were added to sample solution and the relative recovery

of analytes were considered as accuracy of the method. The summarized results are gathered in Table 1. In any cases, the analyte relative recoveries are 100.0 ± 5.0 indicated the method good selectivity for determination of analytes.

Table 1. Effect of interfering ions on relative recovery of analytes in optimum condition.

Cd: 85 ppb, Pb: 85 ppb, Co: 85 ppb.

Interfering ions	(Ion/Cd ²⁺) ratio	RR ^a (%)	(Ion/Pb ²⁺) ratio	RR ^a (%)	(Ion/Co ²⁺) ratio	RR ^a (%)
Cu ²⁺	100	100.2 ± 3.1	100	101.0 ± 2.1	100	102.1 ± 2.3
Ni ²⁺	100	102.0 ± 2.6	100	100.7 ± 3.1	100	103.4 ± 2.5
Hg ²⁺	100	99.1 ± 3.1	100	101.3 ± 2.3	100	101.6 ± 3.3
Zn ²⁺	100	101.6 ± 2.1	100	100.8 ± 2.2	100	101.5 ± 2.9
Fe ³⁺	100	100.9 ± 4.0	100	101.2 ± 3.0	100	99.8 ± 2.4
K ⁺	100	103.0 ± 2.4	100	100.5 ± 2.6	100	100.9 ± 3.4
Ag ⁺	100	102.5 ± 3.0	100	103.8 ± 2.5	100	102.6 ± 2.0
I ⁻	100	101.1 ± 2.1	100	99.1 ± 3.1	100	99.3 ± 2.7
Br ⁻	100	98.0 ± 2.0	100	98.8 ± 2.4	100	99.0 ± 4.0
CO ₃ ²⁻	100	99.1 ± 2.6	100	100.3 ± 3.0	100	99.7 ± 3.1
SO ₄ ²⁻	100	99.9 ± 2.0	100	98.5 ± 2.0	100	100.3 ± 2.4

a: Relative recovery

3.12. Analytical characteristics of method

Calibration graphs were individually plotted for any analytes in optimum condition using standard solution of analytes and the obtained analytical performances were shown in Table 2. All standard deviations were

calculated using 6 replicate measurements (for 20 ppb of each cations). As can be seen, the method have the advantages of low LOD and high enrichment factor which is suitable for determination of analytes at trace levels.

Table 2. Analytical figures of merit for cadmium, lead and cobalt in optimum condition.

Parameters	Cd	Pb	Co
Dithizone-complex λ_{\max} (nm)	495	510	533
Linear range (ppb)	4 - 120	10 - 200	18 - 270
R ²	0.9997	0.9976	0.9999
LOD (ppb), n=6	1.1	3.0	5.4
RSD (%), n=6	3.1	2.9	2.8
Enrichment factor	66.6	65.0	66.0

R²: square of correlation coefficient, LOD: limit of detection, RSD: relative standard deviation

(20 ppb of each cations)

3.13. Analysis of synthetic sample

To evaluate the ability of the proposed method for simultaneous determination of analytes, ternary mixtures of cadmium, lead and cobalt in different proportion were prepared and analyzed by the proposed method. The concentration of analytes in the calibration samples must be non-correlated with each other in order to give the most information from the

analytical system. For the aim, the composition of samples was randomly designed and was shown in Table 1S. Fifteen samples of these ternary mixtures were selected as calibration set to find regression coefficients of analytes spectra with concentration and five samples were chosen as prediction set in order to estimate analyte concentrations and validate the method. All extractions were carried out in the

optimum condition and the recorded spectrophotometric spectra of extracted ternary mixtures of cadmium, lead and cobalt was shown in Figure 3. The resulted spectra were analyzed using Multivar program and the estimated concentrations were presented in Table 2S. The results show that the determination of each analyte concentration in the presence of other analytes is possible and there is good agreement between estimated and real concentration of prediction set.

3.14. Water samples analysis

The proposed method was applied to determination of analytes in tap water, river water, wastewater, well

water and mineral water. For the purpose of investigating the accuracy of the obtained concentration in real samples, the various amounts of cadmium, lead and cobalt were added to real samples. Then, the concentration of analytes in real samples and their relative recoveries in standard added samples were measured using the proposed method. The results are shown in Table 3. The relative recovery of analytes in spiked samples is between 96-103% and falls in acceptable range for water samples at trace levels.

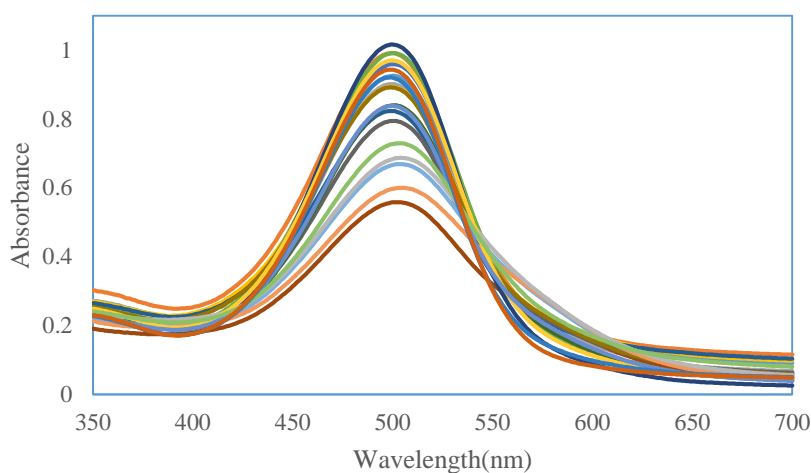


Fig. 3. Plot of the measured spectrophotometric spectra of extracted ternary mixtures of dithizone complexes of Cadmium, Lead and Cobalt. Conditions: aqueous volume: 15 ml, pH: 8, Dithizone: 2 ppm, 1-Undecanol : 150 μ L, Ethanol: 350 μ L, Contact time: 10 min, Stirring rate: 500 rpm, Centrifuging time: 6 min (4500 rpm), Solidification time: 5 min, Freezing temperature: 4 $^{\circ}$ C, Volume of collected extractant: 100 \pm 2 μ L.

Table 3. Determination and recovery of analytes in real samples using NAP/CLS (Each value is the mean of three replicates).

Water Samples	Analyte	Added (ppb)	Found (ppb)	Added (ppb)	Found (ppb)	RR (%)
Tap water ¹	Cd	---	UD	60	59.4	99.0
	Pb	---	UD	80	82.4	103
	Co	---	UD	120	116.4	97
River water ²	Cd	---	UD	60	61.8	103
	Pb	---	UD	80	80.8	101
	Co	---	UD	120	115.2	96
Well water ³	Cd	---	UD	60	58.2	97
	Pb	---	UD	80	79.2	99
	Co	---	UD	120	116.4	97
Wastewater ⁴	Cd	---	6	60	64.8	98
	Pb	---	11.1	80	88.7	97
	Co	---	UD	120	122.4	102
Mineral water ⁵	Cd	---	UD	60	61.8	103
	Pb	---	UD	80	78.4	98
	Co	---	UD	120	115.2	96

¹ Tabriz, Iran; ² Ahar River, Ahar, Iran; ^{3,4} Rajaee Industrial Park region, Tabriz, Iran; ⁵ Sahand Mountain, Shah Spring, Tabriz, Iran. UD:

under detection limit

4. Conclusions

In this study by combining DLLME-SFOD with spectrophotometer instrument equipped with micro liter cells and by recording the absorbance of extracted dithizone complexes, the metal ions were successfully determined at trace levels. The spectra of extracted complexes have sever overlap in whole studied spectra regions. Hence, the multivariate calibrations were used for simultaneous trace ions determination. In DLLME-SFO measurements, the nature and matrix of environmental samples are unknown. Furthermore, the dithizone as general ligand can extract some metal ions; hence, the calibration samples will be dissimilar to prediction samples. In order to resolve this problem, the net analyte signal (NAS) based calibration method was used so as to preprocessing the spectrophotometric data. NAS is a part of signal which is directly proportional to the model predicted concentration. In the current work, the combination of NAS and CLS method, which called NAS/CLS, was used to minimize the un-calibrated interferences in calibration set. Moreover, the mean centering ratio spectra technique was utilized to eliminate the interfering effects of extracted ligand by dividing all spectra to ligand spectrum and then by mean centering the resulted spectrum. The limits of detection were 1.1, 3.0 and 5.4 ppb, relative standard deviations (%) were 3.1, 2.9 and 2.8 % and enrichment factors of the method were 66.6, 65.0 and 66.0 for cadmium, lead and cobalt ions, respectively. In summary, the main benefits of the system were enhancement of instrument sensitivity, minimum organic solvent consumption, removing of matrix constituent, low cost, and high enrichment factor. Finally, the present study may provide a reliable and simple method for cadmium, lead and cobalt ions detection at trace levels in different water samples.

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