

Application of Dispersive Liquid–Liquid Micro-extraction Using Mean Centering of Ratio Spectra Method for Trace Determination of Mercury in Food and Environmental Samples

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Abstract In the present study, dispersive liquid–liquid micro-extraction has been applied for trace extraction and determination of mercury (Hg) ions in environmental samples. The mean centering of ratio spectra method was used to optimize the experimental parameters affecting the extraction of Hg. The factors influencing the extraction procedure such as type and volume of extracting and disperser solvent, concentration of chelating reagent, pH, salt effect, and centrifuge time were investigated and optimized. Under the optimized conditions, the limit of detection of the method was $0.15 \mu\text{g l}^{-1}$ and enrichment factor was 39. The calibration curve was linear in the range of $0.5\text{--}100 \mu\text{g l}^{-1}$ with a correlation of determination (R^2) of 0.998. The relative standard deviation for determination of $40 \mu\text{g l}^{-1}$ of Hg(II) was 2.6 % ($n=5$). The proposed method was applied for the determination of Hg in pine leaf, sea and river fish, sand, and water samples as indicators of environmental pollution and cigarette with satisfactory analytical results. In comparison with other methods, the proposed method is very simple, easy, rapid, and sensitive for determination of Hg at trace levels in complex matrices.

Keywords Mercury · Dispersive liquid–liquid micro-extraction · Mean centering of ratio spectra method · Bioindicator

Introduction

Mercury (Hg) can be taken into the body through food or breathing air. Hg can also be absorbed through the skin, at a lesser extent. Exposure to Hg can cause tremors, memory loss, damage to the brain and kidneys, and changes in personality, vision, and hearing (Argonne National Laboratory 2005). Hg is a naturally occurring metal as a result of the normal breakdown of minerals in rocks and soils from exposure to wind and water and from volcanic activity. The major source of Hg pollution is waste incinerators and coal-fired utilities (Roundhill 2004). Some sophisticated instrumental techniques are extensively used as standard methods in the determination of Hg, but they still possess some disadvantages, such as high cost of instruments, matrix interference, and is time consuming. Spectrophotometry has been most commonly used for quantitative analysis of Hg in solution due to the relatively inexpensive instrumentation and simple operation procedure although its biggest drawback is the low sensitivity which can only detect Hg at parts per million (in milligrams per liter) level normally. However, Hg usually exists in the environment at parts per billion (in micrograms per liter) level with complicated matrix, so extraction and preconcentration procedures are often indispensable (Tuzen and Soylak 2005; Tuzen et al. 2009a, b, c, d).

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So far, a number of techniques have been used for the pretreatment of Hg species from water samples. Although traditional liquid–liquid extraction (Gaona and Valiente 2003) was still employed, new methods such as single-drop micro-extraction (SDME) (Pena-Pereira et al. 2009), liquid–liquid–liquid micro-extraction (Xia et al. 2007), solid-phase extraction (Hashempur et al. 2008) for this purpose have recently been developed. Owing to the low level of trace analytes in samples, these pretreatment approaches play an important role in Hg speciation.

Recently, Assadi et al. (Rezaee et al. 2006) developed a new micro-extraction technique termed dispersive liquid–liquid micro-extraction (DLLME), in which a solvent system containing a mixture of water-immiscible extraction solvent and water-miscible disperser solvent is injected rapidly by a syringe into an aqueous sample. After extraction, the cloudy solution is centrifuged and the enriched analytes in the sediment phase are determined by chromatographic or spectrometric methods (Yousefi and Shemirani 2010; Cheng and Dong 2005; Dadfarnia and Haji Shabani 2010; Bernardo et al. 2010; Yamini and Faraji 2010; Gharehbaghi and Shemirani 2011; Tabrizi 2010; Liang and Sang 2008; Wen et al. 2011; Kocorova et al. 2010; Zhoua et al. 2011).

Spectrophotometric methods are very simple, fast, and inexpensive but lack selectivity and sensitivity. In spectrophotometry, the reagent blank response usually is subtracted from the standard response, when the product and one of the reagents absorb at the same wavelength and hence, the blank absorbance at the end of reaction can be lower than that measured in the beginning as a result of reagent consumption. Therefore, the real absorbance of the product will be larger than the measured value. More recently, a new approach (Afkhami et al. 2007; Afkhami and Bahram 2005; Afkhami and Bahram 2004; Afkhami and Bahram 2006; Afkhami et al. 2005) has been proposed for simultaneous analysis of binary and ternary mixtures called mean centering of ratio spectra. This method uses mathematical equations to give correct responses which can be used for determining analyte concentration which is free from bias and error. This method eliminates derivative steps and therefore signal-to-noise ratio is greatly enhanced (Chamsaz et al. 2010).

The aim of this work was to combine DLLME with ultraviolet–visible (UV–vis) spectrometry to develop an improved procedure for the determination of Hg traces in pine leaf, sea and river fish, cigarette, and water samples with respect to the advantages of DLLME which is a fast and inexpensive method in comparison with other methods of determining Hg. Furthermore, low consumption of organic solvents (at microliter levels) is another advantage of this method, and finally, the authors have gained a good limit of detection (LOD) with a simple instrument like UV–vis spectrophotometer compared with other sophisticated instruments (Gao and Ma 2011). In this work, dithizone

was used as a suitable chelating agent to form complex with Hg and factors that would influence the efficiency of DLLME were also investigated and eventually, mean centering of ratio spectra method was used to eliminate blank bias error.

Experimental

Reagents

All of the reagents used were of analytical grade and purchased from Merck (Darmstadt, Germany). Metal salts, dithizone (1,5-diphenylthiocarbazone), and used extraction and disperser solvents were of analytical grade, and all were purchased from Merck (Darmstadt, Germany). Deionized water was used for all dilutions. Stock metal solution of $1,000 \text{ mg l}^{-1}$ of Hg(II) was prepared by dissolving appropriate amounts of mercuric chloride in 1 % H_2SO_4 . Standard solutions of Hg were prepared daily by appropriate dilution of stock solutions. Dithizone was used without further purification. The reagent solution was prepared daily by dissolving 0.5 mg of dithizone in 10 ml of ethanol.

Instrumentation

Phase separation by centrifugation was used by Centurion Scientific Centrifuge (Model Andreas HettichD72, Tuttlingen, Germany). The pH values were measured with a pH meter (Metrohm, 632) supplied with a glass-combined electrode. A 1.0-ml syringe (Saddle Brook, NJ, USA) was used to inject the mixture of extraction and disperser solvents into the sample solution. A sampler (Eppendorf, Hamburg, Germany) was used to inject the sediment phase into the quartz cell. All spectrophotometric measurements were made with an Agilent 8453 spectrophotometer equipped with a 1-cm path length glass cell. The spectrophotometer was interfaced to a personal computer furnished with the G1115A software.

Mean Centering of Ratio Spectra

Consider a mixture of two compounds X and Y . If there is no interaction among the compounds and Beer's law is obeyed for each compound, it can be written (20):

$$A_m = \alpha_X C_X + \alpha_Y C_Y \quad (1)$$

Where A_m is the vector of the absorbance of the mixture, α_X and α_Y the molar absorptivity vectors of X and Y , and C_X and C_Y are the concentrations of X and Y , respectively. If Eq. (1) is divided by α_Y corresponding to the spectrum of a standard solution of Y in binary mixture, the first ratio

spectrum is obtained in the form of Eq. (2) (for possibility of dividing operation, the zero values of α_Y should not be used in the divisor):

$$B = A_m/\alpha_Y = (\alpha_X C_X/\alpha_Y) + C_Y \quad (2)$$

If Eq. (2) is mean centered since the mean centering of a constant (C_Y) is zero, Eq. (3) would be obtained:

$$MC(B) = MC(\alpha_X C_X/\alpha_Y) \quad (3)$$

The minimum or maximum of these vectors with respect to wavelength is used for the construction of the calibration graph for analyte.

Dispersive Liquid–Liquid Micro-extraction Procedure

One milliliter of $40 \mu\text{g l}^{-1}$ Hg solution at pH of 1.8 was added into a 15-ml test tube with conical bottom. One milliliter of $1.2 \times 10^{-5} \text{ mol l}^{-1}$ solution of dithizone as a chelating agent was added to the solution, and the solution was diluted to 10 ml with deionized water. Then, a mixture of solution containing 750 μl of ethanol (as disperser solvent) and 70 μl of chloroform (as extraction solvent) was rapidly injected into the sample solution using a 2-ml glass syringe. A stable cloudy solution (water, ethanol, and chloroform) appeared, and the mixture was centrifuged for 1 min at 4,000 rpm. The sedimented phase at the bottom of the test tube was separated and diluted with 60 μl of acetone and transferred into a 250- μl cell and its absorbance was measured at 495 nm with UV–vis spectrophotometer. The stored sample spectra were divided by the spectrum of the blank solution according to Eq. (2), and mean centering of these vectors with respect to the wavelength was obtained according to Eq. (3). The minimum or maximum of these vectors with respect to wavelength is used for the construction of the calibration graph for Hg.

Real-Sample Pretreatment for Hg Analysis

Water Samples

Water samples including sea water (Caspian Sea), river water (River Sefidrood), and well water were collected from north of Iran. All the above real-water samples were filtered through an ashless Whatman^R filter paper (40 ashless).

Cigarette

For Hg analysis of cigarette, first, the filter tip of tobacco was removed and 1 g of the sample was dissolved in 25 ml of 18 mol l^{-1} HNO₃ and heated for 24 h, then it was filtered and evaporated to about 4 ml. After that 5 ml of 18 mol l^{-1}

H₂SO₄ was added to the solution and further evaporated until evaluation of white smog to remove HNO₃. The solution was filtered through an ashless Whatman^R filter paper (40 ashless). Finally, the Hg(II) in the cigarette sample was determined by the proposed analytical method (Fan et al. 2008).

Sea and River Fish

Two fish samples were obtained from the Caspian Sea and Sefidrood River, Iran. Each sample was placed in a watch glass, rinsed with water, and dried in an oven at 150 °C. Five grams of dried fish muscle was digested with 6 ml of concentrated HNO₃ (65 %) and 2 ml of concentrated H₂O₂ (30 %) and heated for 10 h. The proteins were precipitated and separated during the digestion process. The resulted solution was filtered through an ashless Whatman^R filter paper for remove the precipitated proteins (40 ashless) (Tuzen et al. 2009a, b, c, d; Nabilid et al. 2012).

Sand Samples

Two sand samples were obtained from the Caspian Sea and River Sefidrood, Iran. Each sample were placed in a watch glass, rinsed with water, and dried in an oven at 50 °C. One gram of dried sand samples were dissolved in 20 ml of a mixture of 12 mol l^{-1} HCl, 14 mol l^{-1} HNO₃, and 18 mol l^{-1} H₂SO₄ and heated for 12 h. The solution was filtered through an ashless Whatman^R filter paper (40 ashless).

Pine Leaf

One gram of pine leaf was dissolved in 25 ml of 18 mol l^{-1} HNO₃ and heated for 24 h, and then it was filtrated and evaporated to about 1 ml. Finally, the Hg(II) in the pine leaf sample was determined by the proposed analytical method.

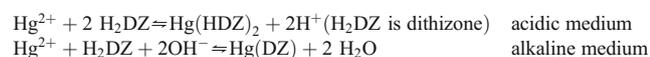
Results and Discussion

As the absorbance spectra of Hg(II)-dithizone complex and dithizone overlap, mean centering of ratio spectra was applied for accurate determination of Hg(II)-dithizone complex. Due to the overlapping of the sample and blank spectra, the blank signal was subtracted from the sample signal. By applying mean centering of ratio spectra (Yousefi and Shemirani 2010; Cheng and Dong 2005; Dadfarnia and Haji Shabani 2010; Bernardo et al. 2010; Yamini and Faraji 2010), the blank signal was eliminated thoroughly and the sample signal was obtained accurately. The effects of different parameters such as pH, type, and volume of the extraction and disperser solvents, centrifuge time, and potential interferences were studied.

Effect of pH

The pH of the sample solution is a very important factor which affects the complex formation of Hg with dithizone. The effect of pH on the Hg complex extraction from water samples was studied in the range of 1–13.5. The results are given in Fig. 1, and as the results show, the optimum pH for extraction of Hg is 1.8. In respect to the complex reaction balance, in pH values lower than 1.8, severe acid effect and the instability of $\text{Hg}(\text{HL})_2$ occur, while high pH values probably cause the hydrolysis of Hg^{2+} and subsequent decomposition of $\text{Hg}(\text{HL})_2$ which finally decreases the absorbance signal, therefore, pH of 1.8 was selected as the optimum pH for further experiments.

Mechanism for the complexation between Hg(II) and dithizone is:



Selection of Extracting Solvent

The type of extracting solvent used in DLLME is of great importance for efficient extraction. In most cases, the ideal extraction solvent has two properties, one is hydrophobicity, and the other one is to have higher solubility for the analytes compared with water. Chloroform (CHCl_3), carbon tetrachloride (CCl_4), dichloromethane (CH_2Cl_2), ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2$), chlorobenzene ($\text{C}_6\text{H}_5\text{Cl}$), and benzyl alcohol ($\text{C}_6\text{H}_5\text{CH}_2\text{OH}$) were studied as extracting solvents. The Hg complex was extracted using 0.5 ml of acetone (disperser solvent) containing 100 μl of each extracting solvents. According to the obtained results, chloroform was selected as the best extracting solvent. Chloroform had higher density

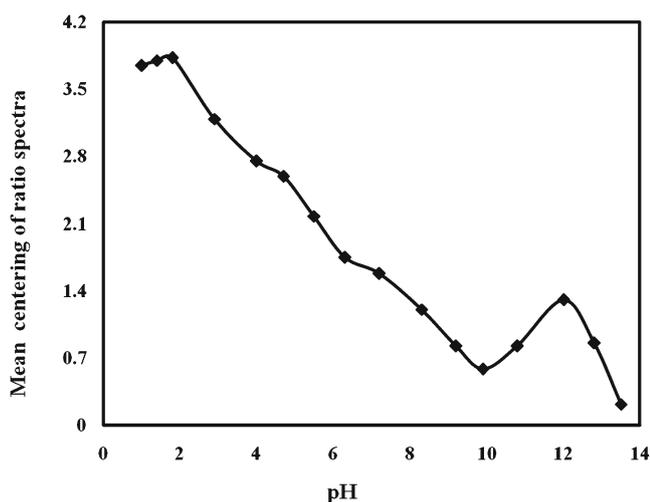


Fig. 1 The effect of pH on absorbance of 40 $\mu\text{g}/\text{l}$ Hg (II). Conditions: 100 μl chloroform, 0.5 ml ethanol, 1.2×10^{-5} mol l^{-1} of dithizone as a chelating agent, and 2 min centrifugation time

than water, increased extracting capability together with low solubility in water and showed higher mean centering of ratio spectra.

Selection of Disperser Solvent

Miscibility of the disperser solvent with extracting solvent and the sample solution is the most important criteria for selection of disperser solvent. Thereby, acetone ($(\text{CH}_3)_2\text{CO}$), acetonitrile (CH_3CN), dimethyl ether (CH_3OCH_3), isopropyl alcohol ($(\text{CH}_3)_2\text{CHOH}$), methanol (CH_3OH) and ethanol ($\text{C}_2\text{H}_5\text{OH}$) were tested as candidates of disperser solvents. The Hg complex was extracted using 0.5 ml of each disperser solvent containing 100 μl of chloroform. The results are shown in Fig. 2. According to the obtained results, ethanol was selected as the best disperser solvent for efficient extraction of Hg(II)-dithizone. Ethanol was soluble in chloroform and was miscible in water. Ethanol enabled to disperse chloroform as fine particles in the aqueous phase and to form a cloudy solution (water/ethanol/chloroform) showing higher mean centering of ratio spectra.

Optimization of Extracting Solvent Volume

In order to obtain the optimum volume of extracting solvent, solutions containing different volumes of chloroform (50, 70, 100, 125, and 150 μl) and 0.5 ml of ethanol were used in DLLME procedure. By increasing of the extraction solvent volume, the final organic phase obtained by centrifugation is increased, resulting in a decrease of the concentration of the target analyte in organic phase. Although the extraction recovery remains almost constant, the enrichment factor will be decreased, leading to a decrease of the sensitivity of the

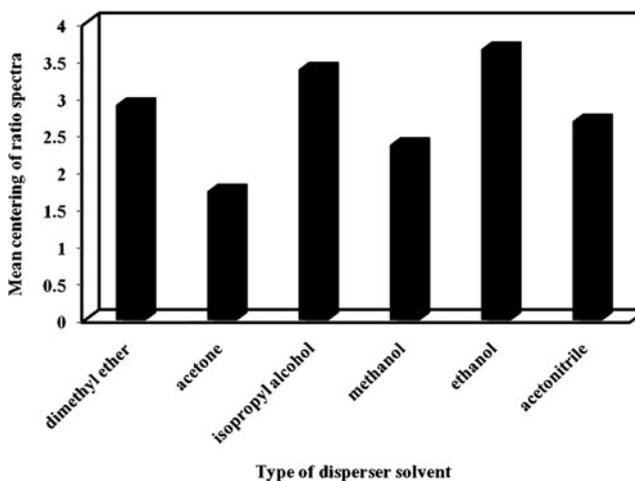


Fig. 2 The effect of type of disperser solvent on absorbance of 40 $\mu\text{g}/\text{l}$ Hg (II). Conditions: 100 μl chloroform, 0.5 ml disperser solvent, pH 1.8, 1.2×10^{-5} mol l^{-1} of dithizone as a chelating agent, and 2 min centrifugation time

determination for the target ions. As the results, the maximum absorbance was obtained using 70 μl of the extracting solvent.

Optimization of Disperser Solvent Volume

The disperser solvent volume directly affects the formation of the cloudy solution (water/disperser solvent/extraction solvent), the degree of the dispersion of the extraction solvent in aqueous phase, and the extraction efficiency. In order to examine the effect of disperser solvent volume, solutions containing different volumes of ethanol (0.25, 0.5, 0.75, and 1 ml) and 70 μl volume of extracting solvent were used in DLLME procedure. The results given in Fig. 3 illustrate that 0.75 ml of disperser solvent was chosen as the optimum disperser solvent volume.

Effect of Centrifuge Time

The effect of centrifuge time on the extraction efficiency was tested. Different centrifuge times (20, 60, 120, 180 s) were used and the results show that the maximum absorbance for Hg(II)-Dithizone was obtained in 60 s as the centrifuge time.

Salt Effect

The effect of ionic strength of the sample solution on the extraction of Hg complex was evaluated by addition of NaNO_3 in the range of 0–0.2 mol l^{-1} into the sample solution containing 40 $\mu\text{g l}^{-1}$ of Hg^{2+} . By addition of salt, an increase in the extraction efficiency was observed up to 0.15 mol l^{-1} NaNO_3 and then it was remained constant.

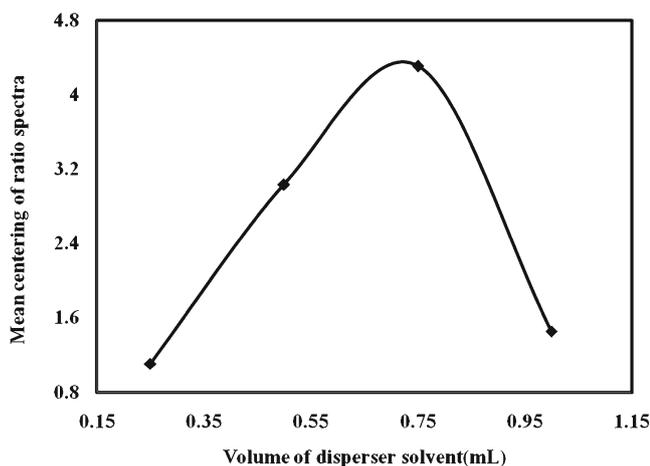


Fig. 3 The effect of volume of disperser solvent on absorbance of 40 $\mu\text{g/l}$ Hg (II). Conditions: 70 μl chloroform, different volume of ethanol, pH 1.8, 1.2×10^{-5} mol l^{-1} of dithizone as a chelating agent, and 2 min centrifugation time

Interferences

Interfering ions could affect the complex formation of Hg(II) ions by their interactions with dithizone. The potential interferences of some ions on the preconcentration and determination of metal ions were examined. To study the effect of interfering ions, 10 ml of the solution containing 40 $\mu\text{g l}^{-1}$ of Hg^{2+} and the interfering ions at 100-fold excess were treated at the optimized conditions followed by the recommended DLLME procedure. Table 1 shows the tolerance limits of the interfering ions. In the present study, the tolerance limit of the coexisting ions is defined as the largest amounts of the coexisting ions that can change the recovery of the metal ions by $\pm 5\%$.

Statistical and Calibration Parameters

The LOD was 0.15 $\mu\text{g l}^{-1}$ based on $3S_b$ for five replicate measurements of blank and the enrichment factors was 39. The linear range was 0.5–100 $\mu\text{g l}^{-1}$ with a correlation of determination (R^2) of 0.998. The relative standard deviation for 40 $\mu\text{g l}^{-1}$ of Hg(II) was 2.6% ($n=5$). Analysis on real

Table 1 Effect of interferences ions on the recovery percent of Hg(II) ions (extraction conditions: 70 μl chloroform, 750 μl ethanol, pH 1.8, 1.2×10^{-5} mol l^{-1} of dithizone as a chelating agent, and 1 min centrifugation time)

Interference	Interference to metal ions ratio (mol/mol)	Recovery (%)
Cl^-	1,000	97.00
CO_3^{2-}	100	91.00
CH_3COO^-	100	92.20
CN^-	100	94.00
HCO_3^-	100	93.00
SO_4^{2-}	100	96.30
NO_3^-	100	97.50
SCN^-	100	100.10
I^-	100	102.30
H_2PO_4^-	100	104.30
Pb^{2+}	100	96.70
Mn^{2+}	100	102.05
Ag^+	100	80.74
AuCl_4^-	100	77.59
Bi^{3+}	100	115.29
Fe^{3+}	100	95.90
Zn^{2+}	100	90.81
Cr^{2+}	100	97.25
Ni^{2+}	100	92.47
Cu^{2+}	100	83.81
Co^{2+}	100	98.35
Cd^{2+}	100	94.06
K^+	1,000	101.04
Na^+	1,000	97.59

Table 2 Determination and relative recovery of spiked Hg in real samples (extraction conditions: 70 μl chloroform, 750 μl ethanol, pH 1.8, 1.2×10^{-5} mol l^{-1} of dithizone as a chelating agent, and 1 min centrifugation time)

Sample	Real sample	Added	Founded	Recovery (%)
Water				
Caspian Sea water	10.42 $\mu\text{g l}^{-1}$	10.00 $\mu\text{g l}^{-1}$	20.80 $\mu\text{g l}^{-1}$	101.8
Sefidrood River water	7.87 $\mu\text{g l}^{-1}$	10.00 $\mu\text{g l}^{-1}$	17.91 $\mu\text{g l}^{-1}$	100.2
Well water	6.41 $\mu\text{g l}^{-1}$	10.00 $\mu\text{g l}^{-1}$	16.52 $\mu\text{g l}^{-1}$	100.6
Sand				
Sea sand	17.72 $\mu\text{g g}^{-1}$	10.00 $\mu\text{g g}^{-1}$	27.23 $\mu\text{g g}^{-1}$	95.1
River Sefidrood sand	12.15 $\mu\text{g g}^{-1}$	10.00 $\mu\text{g g}^{-1}$	21.40 $\mu\text{g g}^{-1}$	96.6
Cigarette	23.80 $\mu\text{g g}^{-1}$	10.00 $\mu\text{g g}^{-1}$	33.40 $\mu\text{g g}^{-1}$	96.0
Fish				
River fish	1.76 $\mu\text{g g}^{-1}$	10.00 $\mu\text{g g}^{-1}$	11.81 $\mu\text{g g}^{-1}$	100.4
Sea fish	3.34 $\mu\text{g g}^{-1}$	10.00 $\mu\text{g g}^{-1}$	13.41 $\mu\text{g g}^{-1}$	100.5
Pine leaf	24.04 $\mu\text{g g}^{-1}$	10.00 $\mu\text{g g}^{-1}$	33.91 $\mu\text{g g}^{-1}$	98.75

samples (River and Sea fish, cigarette, Pine leaf, River and Sea sand, Sea water, well water and River water) were performed and satisfactory results were obtained. Because the amount of Hg in 10 ml of sample solution was measured after preconcentration in a final volume of 0.1 ml, the solution was preconcentrated by a factor of 100. The regression equation was $A=0.118C+0.652$, where A is mean centering of ratio spectra and C is Hg concentration in micrograms per liter.

Analysis of Real Samples

Due to the lack of suitable standard reference materials, the proposed procedure was applied to determine Hg content of water samples, cigarette, pine leaf, river and sea fish, and river and sea sand. The standard addition technique was used for determination of real samples. The results for water, sand, cigarette, fish, and pine leaf samples are given in

Table 2. The recovery of Hg was evaluated using samples spiked with Hg(II) under the optimum conditions. According to the results, the spiked Hg can be quantitatively recovered from all of the samples studied by the proposed procedure. These results demonstrate the applicability of the procedure for mercury determination in real samples.

Comparison of DLLME with the Other Sample Preparation Techniques

A comparison between the presented method and the other reported preconcentration methods for the Hg extraction and determination is shown in Table 3. Some of the preconcentration methods of this table are SDME-ETV-AAS (Bagheri and Naderi 2009), SDME-high-performance liquid chromatography-photodiode array detection (Pena-Pereira et al. 2009), SDME miniaturized spectrophotometer (Yang et al. 2010), carbon nanotube-based sensor (Safavi et al.

Table 3 The comparison between DLLME and other preconcentration techniques for determination of mercury in environmental samples

Method	LOD	RSD	EF	Linear range ($\mu\text{g l}^{-1}$)	References
SDME-ETV-AAS	0.01	6.1	970	0.1–50	Bagheri et al. (2009)
SDME-HPLC-PAD	22.8	11.6	3	80–480	Pena-Pereira et al. (2009)
SDME miniaturized spectrophotometer	0.2	2	69	2–50	Yang et al. (2010)
Carbon nanotube-based sensor	0.64	2.7	–	1.0–30.0	Safavi et al. (2010)
Micro-column-packed GFAAS	0.2	2.8	100	–	Jiang et al. (2006)
Micro-column-packed ICP-OES	0.39	4.8	150	–	Fan (2006)
ETV-ICP-MS	0.12	–	–	0.5–3	Pierre (2012)
Cold vapor AAS	0.10	5	–	–	Brandao et al. (2005)
SPE-cold vapor AAS	0.0025	6	25	–	Tuzen et al. (2009a, b, c, d)
On-line sorption preconcentration CVAFS	0.002	2.2%	35	–	Hong-Jing et al. (2009)
DLLME spectrophotometer	0.15	3.6	39	0.5–100	This work

LOD limit of detection (in micrograms per liter), RSD relative standard deviation (in percent), EF enrichment factor

2010), micro-column-packed GFAAS (Jiang et al. 2006), micro-column-packed ICP-OES (Fan 2006), ETV-ICP-MS (Pierre et al. 2012). Apparently, this work presented a low LOD ($0.15 \mu\text{g l}^{-1}$) and a short extraction procedure with a simple and inexpensive instrument. These characteristics are of great interest for the routine laboratories in the trace analysis of Hg ions.

Conclusions

In the present study, a DLLME was developed for extraction and determination of Hg^{2+} in real samples. DLLME uses an extracting solvent with lower toxicity, and it provides high extraction recovery within a short period of time. The method was successfully applied to determine Hg in environmental samples with high preconcentration factor. Compared with other methods, such as liquid-phase microextraction, DLLME is fast, simple, inexpensive, and consumes small volumes of organic solvents.

Conflict of Interest Mohadese Hossien poor Zaryabi declares that he has no conflict of interest. Mahmoud Chamsaz declares that he has no conflict of interest. Tahereh Heidari declares that he has no conflict of interest. Mohammad Hossein Arbab Zavar declares that he has no conflict of interest. Mohammad Behbahani declares that he has no conflict of interest. Mani Salarian declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

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