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Lead Quantification in Urine Samples of Athletes by Coupling DLLME with UV-Vis Spectrophotometry

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Abstract Urine lead level is one of the most employed measures of lead exposure and risk. The urine samples used in this study were obtained from ten healthy male cyclists. Dispersive liquid–liquid microextraction combined with ultraviolet and visible spectrophotometry was utilized for preconcentration, extraction, and determination of lead in urine samples. Optimization of the independent variables was carried out based on chemometric methods in three steps. According to the screening and optimization study, 133 μL of CCl_4 (extracting solvent), 1.34 mL ethanol (dispersing solvent), pH 2.0, 0.00 % of salt, and 0.1 % *O*,*O*-diethyl dithiophosphoric (chelating agent) were used as the optimum independent variables for microextraction and determination of lead. Under the optimized conditions, R^2 was 0.9991, and linearity range was 0.01–100 μg L^{-1}. Precision was evaluated in terms of repeatability and intermediate precision, with relative standard deviations being <9.1 and <15.3 %, respectively. The accuracy was estimated using urine samples of cyclists as real samples and it was confirmed. The relative error of ≤5 % was considered significant in the method specificity study. The lead concentration mean for the cyclists was 3.79 μg L^{-1} in urine samples. As a result, the proposed method is a robust technique to quantify lead concentrations higher than 11.6 ng L^{-1} in urine samples.

Keywords Dispersive liquid–liquid microextraction · UV-Vis spectrophotometry · Chemometrics · Validation study · Lead · Urine samples of cyclists

Introduction

Lead (Pb) is a heavy metal whose widespread use in diverse industries has led to extensive environmental contamination and health problems [1]. Pb may be absorbed by the body through inhalation, ingestion, or dermal (skin) contact. Once absorbed in the bloodstream, it is primarily distributed among two compartments—the more rapid turnover pool with distribution to the soft tissues such as the liver, lung, spleen, and kidney and the slower turnover pool with distribution to the skeleton [2]. Because the metabolism of lead is affected by many factors, including aging and training status, information on the level of Pb in biological tissue is scarce [3].

Pb toxicity includes adverse effects on the hematological, gastrointestinal, cardiovascular, and renal systems [4]. Also, there are some other effects that have been reported in the literature, including neurotoxicity, carcinogenicity, reproductive toxicity, and neurobehavioral/ developmental effects [5]. Neurotoxicity resulting from lead overexposure is probably the most well-documented effect, particularly in settings involving occupational exposures. Manifestations of Pb toxicity in adults consist of ataxia, memory loss, and at the highest levels, coma, and death. Nerve conduction is reversibly slowed in peripheral nerves at blood lead level of approximately 40 μg dL^{-1} [6]. The US average for blood lead is 2.9 μg dL^{-1}; the Centers for Disease Control and Prevention (CDC) action level (i.e., education and follow-up testing) is

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10 μg dL^{-1}, and the intervention level (clinical case management) is 20–44 μg dL^{-1}. For children 1–2 years of age, the most recent data show that the mean level is 3.1 μg dL^{-1} [5]. According to the World Health Organization (WHO) report, human exposure to lead is estimated to account for 143,000 deaths every year and 0.6 % of the global burden of disease [7]. Therefore, as a notoriously toxic element, which is invariably present in our society, lead is a major public health concern and has attracted a great interest in its determination.

Atomic absorption spectrometry equipped with flame (FAAS) and graphite furnace (GFAAS) atomization system [8, 9], inductively coupled plasma hyphenated with mass spectrometry (ICP-MS) and atomic emission spectrometry (ICP-AES) [10, 11], are well-known analytical methods for the quantification of trace levels of lead in different samples. Despite the significant progress in analytical instrumentation in recent decades, at least a sample pretreatment step is still often required prior to performing a determination of lead at trace levels in complex matrices. Liquid–liquid extraction (LLE) [12] and solid-phase extraction (SPE) [13] were widely used for separation and preconcentration of trace lead, but some drawbacks, such as the need for large sample volumes and toxic organic solvents, time consuming, the emulsion formation, unsatisfactory enrichment factors, and secondary wastes, restrict their applications. Therefore, the miniaturized alternatives of conventional methods were developed to improve the shortcomings [14–18].

In 2006, Rezaei and colleagues introduced a rapid, economic, easy-to-operate, and eco-friendly sample pretreatment technique known as dispersive liquid–liquid microextraction (DLLME) [19]. This technique represents one of the most popular sample preparation procedures, which has been widely applied for analysis of organic and inorganic analytes in different matrices [20]. DLLME can be jointed with different atomic and molecular spetrochemical techniques either independently or in combination with a separation technique, as well as hyphenated technologies [21]. Ultraviolet and visible (UV-Vis) spectrophotometry is a fundamental analytical tool utilized in different scientific areas, such as analytical chemistry, microbiology, and biochemistry, due to its simplicity, flexibility, ease of operation, low cost, and convenience [22]. The combination of DLLME with UV-Vis spectrophotometry is a difficult task due to the low volume of solution resulting from DLLME and the need to focus the light for studying microliter volumes. Nevertheless, recent advances in electronics and optics have led to the invention of new generations of miniaturized spectrophotometric systems to determine the metallic [23–30] and non-metallic [31–34] species. A brief review of the literature indicates that the problem of small volumes in coupling DLLME with UV-Vis spectrophotometry has often been resolved by evaporation and/or dilution of the sedimented phase remaining after extraction, which led to serious drawbacks such as employing an additional step into the procedure and a decrease in the sensitivity of determination. While the number of publications on DLLME has seen an exponential growth in recent years, the abovementioned shortcomings of the method call for efforts to develop combined approaches [35].

The aim of this study was to couple DLLME method with the UV-Vis spectrophotometry for lead preconcentration, microextraction, and quantification in urine samples of cyclists and to evaluate the effects of various experimental variables to optimize the procedure by focusing on the chemometrics. Consequently, the method was followed by a validation step.

Experimental

Reagents

All the chemicals and solvents, either supra-pure or with analytical reagent grade, were purchased from Merck (Darmstadt, Germany). Appropriate amount of lead(II) nitrate was dissolved in ultra-pure water to prepare 1000 μg mL^{-1} Pb stock solution. The working solutions were prepared daily by diluting the stock solution with ultra-pure water. All water used in these experiments was supplied by the Milli-Q RG ultra-pure water system (Millipore, USA). To establish the validity of our results, Clincheck control-lyophilized human urine (Recipe, Munich, Germany) was used as certified reference materials (CRM).

Apparatus and Software

A Shimadzu UV-Vis 1601PC double beam spectrophotometer (Kyoto, Japan) controlled by a UVPC Personal Spectroscopy Software version 3.91 and equipped with a 70-μL quartz cell was used for reading the absorbance. Spectra were obtained within the range of 300–550 nm (1 nm resolution). Centrifugation was carried out using a Behdad Universal centrifuge (Tehran, Iran). A Metrohm pH meter (Herisau, Switzerland) equipped with a combined electrode was used to determine the pH value. Design of experiments and data analysis was performed and evaluated using Minitab, version 17.

Subjects

Ten healthy male cyclists (age, 22 ± 2) who lived in Tehran city were recruited before the start of their training period. The athletes had performed training regularly for the previous 2 years, and they have an average of three times per week work out, mostly on city streets. All the participants were informed about the purpose of the study and gave informed consent. They all completed a questionnaire relative to their

habits to ensure they were not taking any vitamins, minerals, or other supplementations. Experimental procedures were approved by the Ethics Committee of Islamic Azad University, Varamin-Pishva Branch.

Urine Collection

This cross-sectional study was conducted in spring 2016. Morning midstream urine samples obtained from all subjects were collected in polystyrene urine collection bottles (Sterilin, Newport, UK) previously washed with diluted nitric acid and frozen at −20 °C until analysis. Prior to analysis, the samples were thawed and homogenized by shaking.

Dispersive Liquid–Liquid Microextraction Procedure

The microextraction of lead ultra-trace quantity from a urine sample into a microvolume of the organic solvent by DLLME method was based on complexation with *O*, *O*-diethyl dithiophosphoric (DDTP) acid. Five milliliters of diluted sample containing 10.0 μg L^{-1} Pb(II), with the pH adjusted to 2, was pipetted into a 30-mL screw-capped glass test tube with a conical bottom. Then, 750 μL of methanol (dispersing solvent), containing 70 μL of carbon tetrachloride (extracting solvent) as well as 5 μL DDTP (chelating agent), was vigorously injected into the solution by a 2-mL glass microsyringe (Hamilton, Reno, NV, USA) to generate a cloudy state. The mixture was centrifuged at 3000 rpm for 2 min, and the resulting rich sediment phase of ≈100 μL, comprising the complex DDTP-Pb(II) soluble in CCl_4, was collected by a convenient microsyringe and transferred into a matched quartz microcell for the complex determination at 293 nm.

Results and Discussion

Optimization of DLLME Procedure

The first step in the optimization procedure was to select the appropriate independent variables. Based on the preliminary tests and previous reports on DLLME method, seven parameters were evaluated, including type and volume of extracting and dispersing solvents, pH, ionic strength, and chelating agent concentration. A classical univariate method and then a two-step design (screening and optimization) were utilized for searching the optimal type of extraction and disperser solvents and other main factors.

Selection of Extracting and Dispersing Solvents

The extracting and dispersing solvents used are the two of the most important factors influencing the extraction efficiency [36]. In this study, several solvents such as chloroform, carbon tetrachloride, tetrachloroethylene, and carbon disulfide (as extracting solvent) and methanol, ethanol, acetone, and acetonitrile (as dispersing solvent) were investigated. The experiments were performed using 2.0 mL of dispersing solvent containing 30 μL of the extracting solvent and 5 μL of DDTP. Different combinations of extracting and dispersing solvents were compared for lead preconcentration. A two-way analysis of variance (ANOVA) was conducted to examine the influence of two independent variables, i.e., extracting and dispersing solvent type, on the extraction efficiency. Both independent variables included four levels. All effects were significant at the level of $P = 0.05$. There were significant main effects for the extracting and dispersing solvent types, $F(3, 32) = 7164.7$, $P < 0.001$ and $F(3, 32) = 1270.9$, $P < 0.001$, respectively. The interaction effect between extracting and dispersing solvent types was significant, $F(9, 32) = 211.5$, $P < 0.001$. Post-hoc comparisons using the Bonferroni test indicated that ethanol (as dispersive solvent) and carbon tetrachloride (as extraction solvent) had maximum extraction efficiency.

Screening Design

In the next step, a fractional factorial design (FFD) $2^{(5-2)}$ was employed for screening the significant variables (i.e., volume of extracting and dispersing solvent, sample pH, ionic strength, and chelating agent concentration) and assessing their effects on the performance of the methods through a multivariable approach. Eventually, a total number of 20 experiments (including four replicates at central points to estimate the experimental error) were randomly performed in order to nullify the effect of extraneous or "nuisance" variables. Details of the experimental design containing the code used and the low and high levels of each factor are displayed in Table 1. The analysis of variance was performed on the design to evaluate the significance of the model. Analysis of the results of the ANOVA demonstrated that the model terms are significant (Table S1). Therefore, beside the main effects of A, B, C, and D, the two-factor interactions of DE and CE are also statistically significant (Fig. 1a). The negative effect of ionic strength (C), and pH (D) on the absorbance of lead was identified in the normal plot (Fig. 1b); thus, the subsequent experiments were performed in pH = 2.0, without salt addition. Two-level designs only support linear models of responses and are unable to provide information about maxima or any non-linear relationships. Therefore, a design with more levels was necessary.

Optimization Design

Finally, the variables with significant influence were optimized, and the detailed dependence of different factors on a response was consequently identified using a central composite design (CCD) [37]. The number of experiments (N_t)

Table 1 Experimental variables, levels, design matrix, and results (absorbance arbitrary units) in the $2^{(5-2)}$ fractional factorial design

Variables					Coded	Levels		
						Low (−1)	Center (0)	High (+1)
Volume of CCl_4 (extracting solvent) (μL)					A	40	75	110
Volume of methanol (dispersing solvent) (mL)					B	1.0	2.5	4.0
pH value					C	2	4	8
Concentration of NaCl (ionic strength; *w*/*v*) (%)					D	0	5	10
Concentration of DDTP (chelating agent; *v*/*v*) (%)					E	0.1	0.3	0.5
Run	Order	Block	A	B	C	D	E	Abs.
1	6	1	−1	+1	−1	+1	+1	0.540
2	1	1	+1	−1	−1	−1	−1	1.119
3	8	1	−1	+1	+1	+1	−1	0.357
4	4	1	−1	+1	+1	−1	+1	0.664
5	10	1	0	0	0	0	0	0.360
6	5	1	+1	−1	−1	+1	+1	0.765
7	9	1	0	0	0	0	0	0.350
8	3	1	+1	−1	+1	−1	+1	0.612
9	2	1	−1	+1	−1	0	−1	0.847
10	7	1	+1	−1	+1	+1	−1	0.411
11	20	2	0	0	0	0	0	0.340
12	12	2	+1	+1	−1	−1	+1	0.851
13	17	2	−1	−1	+1	+1	+1	0.172
14	13	2	−1	−1	+1	−1	−1	0.311
15	14	2	+1	+1	+1	−1	−1	0.912
16	19	2	0	0	0	0	0	0.349
17	11	2	−1	−1	−1	−1	+1	0.304
18	16	2	+1	+1	−1	+1	−1	0.743
19	15	2	−1	−1	−1	+1	−1	0.469
20	18	2	+1	+1	+1	+1	+1	0.586

needed for the development of this design is defined as $N_t = N^f + N_a + N_0$, where N is the number of levels of factorial design, f is the number of factors, N_a ($2f$) is the star points, and N_0 is the points at the center of the experimental region, which are usually repeated to achieve a good estimation of experimental error [38]. Based on the preliminary studies and experiments, two variables, namely volumes of extracting and dispersing solvents (A and B), at low, central, and high levels were evaluated. The factors, their levels, symbols, and design matrix for CCD are demonstrated in Table 2. The star points were placed at $+a$ and $-a$ from the center of the experimental domain. The value of "a" required to ensure orthogonality and rotatability was selected equal to ±1.414. The runs at the center of the experimental field were carried out three times more. In this study, overall, the matrix of CCD included 13 experiments, which were carried out in random order.

Analysis of variance was used to evaluate the statistical significance of the model (Table 3). The experimental data, according to P value, revealed that the regression and the lack of fit (LOF) of the model were significant and insignificant, respectively. In this case, A and B were significant model terms. Therefore, a second-order polynomial model was adopted, which depicted the correlation between dependent and independent variables. This model, in terms of coded level variables, could be declared by Eq. 1:

$$Y = -\beta_0 + \beta_1 A + \beta_2 B - \beta_{11} A^2 - \beta_{22} B^2 - \beta_{12} AB \quad (1)$$

where Y is the dependent variable, β_0 is the intercept term, A and B are the independent variables, β_1 and β_2 are the linear coefficients, β_{11} and β_{22} are the quadratic term coefficients, and β_{12} is the cross product coefficients. It contains two main effects, two two-factor interaction effects and two curvature effects. The response surface method (RSM) was employed to obtain the empirical relationships between response (Y) and the independent variables by using multiple regression analysis (Eq. 2):

$$Y = -0.2467 + 4.316\text{e}{-3}\, A + 1.658\text{e}{-3}\, B - 1.2\text{e}{-5}\, A^2 - 1.0\text{e}{-6}\, B^2 - 1.0\text{e}{-6}\, AB \quad (2)$$

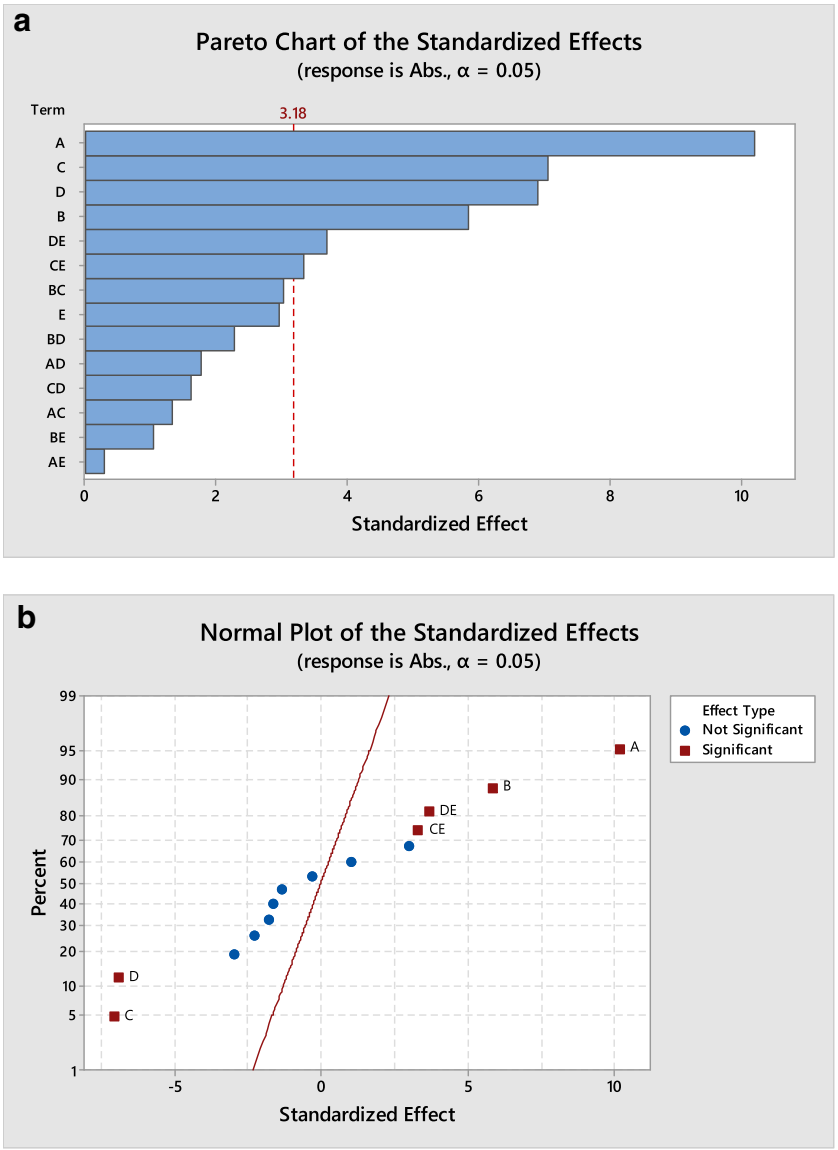

Fig. 1 **a** Pareto chart of the standardized effects at $P = 0.05$. The *vertical line* defines the 95 % confidence interval. *A* volume of extracting solvent, *B* volume of dispersing solvent, *C* pH value, and *D* ionic strength. **b** Normal probability plot of standardized effects at $P = 0.05$

The quality of fit of the polynomial model equation was explained by the coefficient of determination. Values for R^2 (0.9960) as a measure of the variation around the mean and adjusted R^2 (0.9931) as an adjustment for the number of terms in the respective model evidenced a good correlation between the experimental data and the fitted model. To provide a graphical representation of the interactions, a three-dimensional two-factor response surface plot of the model was developed (Fig. 2). Results showed that increasing the volume of the extracting solvent from 32 to 150 μL increased the absorbance, but increase from 150 to 287 μL was associated with decreased absorbance. On the other hand, it is obvious that the absorbance increases when increasing the volume of dispersing solvent up to 1300 μL and then decreases. Accordingly, for low volumes of dispersing solvent (EtOH) and extracting solvent (CCl_4), the absorbance is low (<0.5). This may be ascribed to the fact that the low volume of ethanol leads to a lack of effective dispersion of CCl_4 and consequently, cloudy solution was not completely formed. For low amounts of ethanol, the absorbance increased gradually with increasing CCl_4, reaching a maximum. However, at higher volumes of CCl_4 (>1300 μL), excess ethanol affected the absorbance negatively. This may be ascribed to the fact that higher amounts of dispersing solvent increase the solubility of the target analyte, leading to reduced extraction efficiency. The highest absorbance ($R \approx 86$ %) was observed in the region where low to moderate volumes of CCl_4 (0.4–0.8 mL) and high volumes of ethanol (200–250 μL) were utilized as extracting and dispersing solvents, respectively.

Therefore, according to the screening and optimization study, 133 μL of CCl_4 (extracting solvent), 1.34 mL ethanol (dispersing solvent), pH 2.0, 0.00 % of salt, and 0.1 % DDTP (chelating agent) were chosen as the optimum independent variables for microextraction and determination of lead.

Table 2 Experimental variables, levels, design matrix, and results (absorbance arbitrary units) in the central composite design

Variables		Coded	Levels			Star point (a = 1.414)	
			Low (−1)	Center (0)	High (+1)	−a	+a
Extracting solvent volume (μL)		A	70	160	250	32.7	287.3
Dispersing solvent volume (μL)		B	500	1000	1500	292.9	1707.1
Run	Order	Block	A	B	Abs.		
1	2	1	+1	−1	0.65		
2	4	1	+1	+1	0.95		
3	3	1	−1	+1	1.08		
4	11	1	0	0	1.09		
5	10	1	0	0	1.08		
6	8	1	0	+a	1.05		
7	13	1	0	0	1.09		
8	7	1	0	−a	0.55		
9	6	1	+a	0	0.84		
10	5	1	−a	0	0.95		
11	1	1	−1	−1	0.63		
12	9	1	0	0	1.08		
13	12	1	0	0	1.06		

Validation of the Method Analytical Parameters

One of the main objectives of this study was to develop a new analytical procedure that could reliably quantify lead in urine samples using DLLME coupled with UV-Vis spectrophotometry. Validation of method is one of the most important aspects in analytical chemistry to guarantee the quality and trustworthiness (the reliability, traceability, or comparability) of the results. According to the International Conference on Harmonization (ICH) and International Union of Pure and Applied Chemistry (IUPAC) guidance documents, the characteristics to be considered in the validation of a quantitative procedure are calibration model (linearity), range, detection limit, quantitation limit, precision (repeatability and intermediate precision), accuracy, and specificity [39, 40].

Statistical Validation of a Calibration Model

In order to select an appropriate calibration model, the relationship between the ingredient concentration in certified reference urine sample and the corresponding response (absorbance) was investigated. Calibration curve was obtained in the concentration range between 0.001 and 100 μg L^{-1}, using ten calibration standards (0.001, 0.01 0.1, 0.5, 1.0, 5.0, 10.0, 20.0, 50.0, and 100.0 μg L^{-1} Pb) prepared individually under the same conditions. Each concentration was measured in triplicate under the optimized sample pretreatment conditions. The fitness of the model equation for regression line (y = 0.0211x + 0.2457) was estimated by the correlation coefficient (R). The value of R (0.9991) clarified good relationship between the independent variables and the response and also demonstrated that the calculated model was able to represent 99.91 % of the results.

Table 3 Analysis of variance for the fitted quadratic polynomial model of microextraction of lead in urine sample

Source	DF	SS	MS	P value
Model	5	0.469803	0.093961	0.000
Regression	2	0.274211	0.137105	0.000
Lack of fit	3	0.001289	0.000430	0.168
Pure error	4	0.000600	0.000150	

DF degrees of freedom, *SS* sum of squares, *MS* mean squares

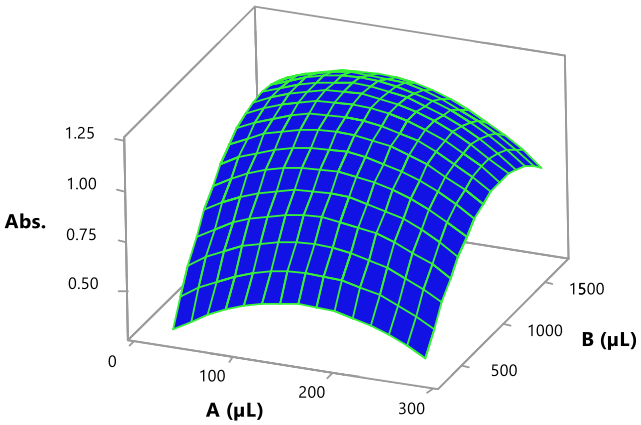

Fig. 2 Three-dimensional (3D) response surface estimated from the central composite design for volume of extraction solvent (**a**) versus volume of disperser solvent (**b**)

Linearity and Range

Linearity, as an important attribute in the development of an analytical method, is usually evaluated by calculating the correlation coefficient (R) of a calibration graph, which may lead to misconception by the unwary and is encouraged to be avoided [41, 42]. In our study, linearity was assessed using ANOVA. Consequently, the ratio of the lack-of-fit (LOF) variance to the squared pure error was calculated through a statistic F test. Since the calculated value of F (7, 18) = 2.34 was less than the critical value, F (7, 18) = 2.58, the null hypothesis of the test for significance of the regression was accepted (the model was significant) at 5 % level (Table S2). Also, the P value of greater than 0.05 approved the statistical significance of the model. According to the data obtained from calibration plot, linearity, and accuracy studies, the valid analytical range of the method was linear from 0.01 to 100 μg L^{-1}.

Sensitivity

Similar to linearity, analytical sensitivity is affiliated with calibration graph and is usually defined as the change in the response of a measuring instrument divided by the corresponding change in the stimulus [43] or, alternatively, as the gradient of the calibration function [40]. Although analytical sensitivity is not recognized as an essential parameter in some method validation guidelines (e.g., ICH), it is very significant in method optimization and regular monitoring of the instrument [39]. Anyway, the limit of detection (LOD) and the limit of quantitation (LOQ) are the two parameters, which are frequently used to assess the analytical sensitivity. In this study, sensitivity was utilized as defined by IUPAC [40].

Limit of Detection and Limit of Quantification

The IUPAC's modern recommendation to calculate the LOD was expressed as Eq. 3:

$$\mathrm{LOD} = \left(3.3 s_{y/x}\right)/A\sqrt{(1 + h_0 + 1/I)} \quad (3)$$

where $s_{y/x}$ is the residual standard deviation, A is the slope of the univariate calibration graph, I is the number of calibration samples, and h_0 is the leverage for the blank sample:

$$h_0 = \frac{\overline{c}_{\mathrm{cal}}^{\,2}}{\sum_{i=1}^{I}\left(c_i - \overline{c}_{\mathrm{cal}}\right)^2} \quad (4)$$

where c_{cal} is the mean calibration concentration and c_i is each of the calibration concentration values. Other equations, e.g., Eq. 5 below, have been used for calculations of LOQ:

$$\mathrm{LOQ} = \left(10 s_{y/x}\right)/A\sqrt{(1 + h_0 + 1/I)} \quad (5)$$

where the factor 10 ensures a maximum relative prediction uncertainty of 10 %. The LOD and the LOQ computed according to the modern definition were 3.6 and 11.6 ng L^{-1}, respectively.

Precision

The precision of the method, in terms of "repeatability" and "intermediate precision," was evaluated by obtaining the relative standard deviation (RSD) of the spiked reference urine samples at low, medium, and high concentration levels.

Repeatability

Repeatability refers to precision under the same operating conditions over a short interval of time. It is also termed intra-assay precision or intra-day precision [39]. To evaluate the repeatability, we carried out 21 determinations in the same day (three concentrations, seven replicates each) by microextracting urine samples spiked at 0.5, 5.0 and 50.0 μg L^{-1}. RSDs were lower than 9.1 % at the low, medium, and high concentration levels (Table S3).

Intermediate Precision

Intermediate precision, also known as inter-assay precision or inter-day test, measures the effects of within-laboratory random events such as different days, different analysts, and different equipment on the precision of the analytical procedure [39]. To study the intermediate precision, two [independent] analysts performed three independent DLLME-UV-Vis analyses and 27 determinations (three levels of concentration, three determinations per day for 3 days). ANOVA was also performed on the data obtained during 3 days to evaluate intermediate precision ($P > 0.05$, 95 %). The results presented in Table S3 meet the common quality control criteria with an RSD of <15.3 % [44, 45].

Accuracy

Accuracy is often used to describe the systematic error component and bias and is commonly assayed by using recovery experiments. In this study, the recovery experiments were performed by addition of known amounts of the standard solution

to the matrix in 1.0, 5.0 and 15 μg L^{-1}, three replications each. Accordingly, for each concentration level, the accuracy was reported as percent recovery by the assay of known added amount of the analyte in the sample or as the difference between the mean and the accepted true value together with the confidence intervals. Finally, the results were statistically evaluated by comparative analysis (*t* test), at 95 % level of confidence, organizing the null hypothesis as H_0: $\overline{R}_{exp}$ = 100 % and the alternative hypothesis as H_1: $\overline{R}_{exp} \neq 100$ % [41]. The experimental t_{exp} value was computed by the following:

$$t_{exp} = |100 - \left(\overline{R}_{exp} | \sqrt{N}\right) \Big/ S_R \quad (6)$$

Table 4 The accuracy of proposed method in four urine samples of cycling athletes and recovery analysis using Student's *t* test ($n = 3$)

Sample	Spiked (μg L^{-1})	Found (μg L^{-1})	Recovery (%)	Trueness bias (%)	RSD (%)	t_{exp}
Urine sample1	0	0.90	–	–	–	
	1.0	1.92	102.0	2.0	2.8	1.26
	5.0	5.87	99.4	−0.6	4.5	
	15.0	15.96	100.4	0.4	3.7	
Urine sample2	0	3.85	–	–	–	
	1.0	4.83	98.0	−2.0	2.5	1.02
	5.0	8.90	101.0	1.0	4.2	
	15.0	18.88	100.2	0.2	3.8	
Urine sample3	0	3.79	–	–	–	
	1.0	4.81	102.0	2.0	4.9	1.22
	5.0	8.77	99.6	−0.4	5.4	
	15.0	18.74	99.7	−0.3	3.5	
Urine sample4	0	5.89	–	–	–	
	1.0	6.87	98.0	2.0	5.1	0.89
	5.0	10.90	100.2	0.2	4.6	
	15.0	21.17	101.9	1.9	2.8	
Urine sample5	0	1.75	–	–	–	
	1.0	2.76	101.0	1.0	3.1	1.12
	5.0	6.73	99.6	−0.4	2.7	
	15.0	16.98	101.5	1.5	3.9	
Urine sample6	0	4.64	–	–	–	
	1.0	5.67	103.0	3.0	5.2	0.92
	5.0	9.70	101.2	1.2	3.1	
	15.0	19.11	96.5	−3.5	3.5	
Urine sample7	0	2.77	–	–	–	
	1.0	3.75	98.0	−2.0	2.8	1.24
	5.0	7.75	99.6	−0.4	4.0	
	15.0	17.91	100.9	0.9	3.8	
Urine sample8	0	6.89	–	–	–	
	1.0	7.91	102.0	2.0	4.5	1.19
	5.0	11.96	101.4	1.4	2.4	
	15.0	21.41	96.8	−3.2	3.3	
Urine sample9	0	2.69	–	–	–	
	1.0	3.66	97.0	−3.0	5.6	1.41
	5.0	7.81	102.4	2.4	3.9	
	15.0	17.86	101.1	1.1	3.2	
Urine sample10	0	4.75	–	–	–	
	1.0	5.77	102.0	2.0	4.7	1.94
	5.0	9.88	102.6	2.6	5.1	
	15.0	19.56	98.7	−1.3	4.9	

$t_{(0.025,\ 8)} = 2.30$

where $\overline{R}_{exp}$ is the average experimental recovery, N the sample size, and S_R the standard deviation of the recoveries.

The results (Table 4) showed no statistical difference ($P > 0.05$); hence, $t_{exp} < t_{(0.025,N-1)}$, which confirms the accuracy of the method.

The lead concentration median and mean in urine samples of cyclists studied in this research were 3.82 (range from 0.9 to 6.89) and 3.79 μg L^{-1}, respectively. Comparing median value to the results of Berdahl et al. in 1997 with urine lead concentration median and range of 10 (2.0–176) μg L^{-1} for exposed workers shows that the value of our subjects' urine lead is lower, which more likely indicates they had lower exposure levels compared to Berdahl study [46].

Specificity

In this study, specificity was investigated by evaluating the effects of common coexisting ions on DLLME of lead. The sample solution was fortified with varying amounts of different ions and analyzed under the proposed optimized procedure. The relative error ≤ 5 % was considered as the tolerance limit, expressed as the maximum concentration of the coexisting ion, leading to a 5 % variation in the analytical signal of target analyte (Table 5). DDTP, as the complexing agent, has stability in strong acidic media. Hence, there is no need to use buffers, which are sources of impurity per se. The results demonstrated that DDTP is quite selective, and since it does not form complexes with alkaline and alkaline earth elements, it allows the determination of lead in urine samples and high-salt matrix samples. Ions such as Mn, V, Ti, Cr, Zn, Al, and several other elements do not interfere at concentrations up to at least 1000 μg L^{-1}. As Table 5 demonstrates, the major interferences in Pb determination are Cu^{+2}, Fe^{+3}, sulfite, and sulfate. When extracting Pb-DDTP from acidified solution (pH ≈ 2) containing Cu^{+2}, it was recognized that cupper ion formed Cu-DDTP, which led to low recoveries of the main complex. To overcome the copper ion interference, we studied the effect of various masking agents and pH values. The results showed that addition of cyanide ion at neutral pH (≈5) resulted in the formation of cuprocyanide, which is sufficiently stable for quantitative recovery of Pb-DDTP to be achieved. The presence of ferric ion is unfavorable because it promotes the degradation of DDTP through a redox reaction. This interference can be overcome by reducing Fe^{+3} to Fe^{+2} by adding ascorbic acid before complexation, as the latter ion does not complex with DDTP. In acidified solutions, sulfite or thiosulfate ion decomposes to sulfur dioxide, which can be extracted by carbon tetrachloride and also yields a broad absorption maximum at about 288 nm. This wavelength is close to that of Pb-DDTP, so it can introduce disturbance to determination of the target analyte. The results indicated that quantitative extraction of Pb-DDTP was attained from a solution including sulfite at neutral pH [21, 22]. Another possible interference is sulfate ion, which can result in the precipitation

Table 5 Effect of diverse ions on the recovery of 50 μg L^{-1} Pb(II) in urine sample using DLLME-UV-Vis

Diverse ion	Concentration (μg L^{-1})	Ion:Pb(II) ratio	Recovery (%)	Relative error (%)
Na^{+}	50,000	1:1000	100.0	0.03
K^{+}	50,000	1:1000	104.4	4.4
Ca^{+2}	50,000	1:1000	103.3	3.2
Mg^{+2}	50,000	1:1000	96.8	−3.3
Ba^{+2}	50,000	1:1000	95.7	−4.3
Mn^{+2}	25,000	1:500	97.9	−2.1
Al^{+3}	25,000	1:500	102.2	2.2
Zn^{+2}	25,000	1:500	103.6	3.6
Cr^{+3}	25,000	1:500	101.3	1.3
Sn^{+2}	25,000	1:500	104.7	4.7
V^{+5}	25,000	1:500	98.7	−1.3
Ni^{+2}	10,000	1:200	99.0	1.0
Co^{+2}	7500	1:150	103.2	3.2
Cd^{+2}	5000	1:100	97.0	−3.0
Hg^{+}	1000	1:20	104.9	4.9
Ag^{+}	500	1:10	97.4	−2.6
Bi^{+2}	500	1:10	105.0	5.0
Cl^{-}	50,000	1:1000	97.9	−2.1
NO_3^{-}	50,000	1:1000	104.9	4.9
CH_3COO^{-}	25,000	1:500	103.5	3.5
$Cr_2O_7^{-2}$	25,000	1:500	98.9	−1.1
Cu^{+2}	50	1:1	95.7	−4.3
	1000	1:20	62.3	−37.7
	1000[a]	1:20	98.6	−1.4
Fe^{+3}	50	1:1	96.1	−3.9
	2000	1:40	53.9	−46.1
	2000[b]	1:40	101.3	1.3
SO_4^{-2}	100	1:2	97.5	−2.5
	2500	1:50	73.6	−26.4
	2500[c]	1:50	99.1	−0.9
SO_3^{-2}	100	1:2	99.5	−0.5
	2500	1:50	112.8	12.8
	2500[d]	1:50	101.2	1.2

[a] CN^{-} (30 μg L^{-1}), pH = 4.7

[b] Ascorbic acid (40 μg L^{-1})

[c] Barium perchlorate (50 μg L^{-1})

[d] pH ≈ 5

Table 6 Robustness evaluation of the proposed method

Variable	Studied range	Recovery (%)	RSD% (n = 3)	P value
Wavelength (nm)	291	100.0	0.15	0.13
	293	99.4	0.81	
	295	98.7	0.97	
Centrifugation time (min)	10	99.5	0.91	0.15
	15	99.4	0.81	
	20	100.8	0.40	
Temperature (°C)	30	100.5	100.5	0.05
	23	99.4	99.4	
	4	98.3	98.3	

Table 7 Comparison of the characteristics of procedures for determination of lead with the proposed method in this study

Preconcentration method	Detection method	Linearity (μg L^{-1})	LOD (ng L^{-1})	RSD (%)	Sample volume (mL)	Time (min)	Reference
LLE	ICP-MS	0.2–0.5	17	4.5	5.0	20	[12]
SPE	GFAAS	0.1–10.0	12	3.2	>3.3	20	[13]
CPE	GFAAS	1.0–30.0	80	2.8	10.0	30	[16]
DLLME	GFAAS	0.1–2.0	10	2.4	5.0	–	[17]
IL-DLLME	GFAAS	0.01–0.4	3.0	7.3	10.0	<6	[18]
DLLME	UV-Vis	0.01–100	3.6	9.1	5.0	<5	This method

of lead sulfate. Barium perchlorate, as a masking agent, has been shown to be a proper precipitant to eliminate the bulk of any sulfate present in the sample. To investigate the presence of interferences in an unknown situation, we recommend performing preliminary experiments on the original urine solution.

Robustness

To evaluate the robustness of the proposed method, the experimental conditions such as centrifugation time (±5 min), wavelength of measurement (±2 nm), and temperature were manipulated and the results were assessed. The ANOVA test was also performed on data obtained from small changes of the variables to evaluate the method robustness ($P > 0.05$, CI = 95 %). Results indicated no significant differences within the limits [Table 6].

Comparison with Other Methods

The analytical figures of merit for the DLLME-UV-Vis analysis of lead are compared with other extraction methods in Table 7. The results illustrate that proposed technique is an improvement over earlier reported methods with significant merits. The limit of detection, linear range, and sample volume of present method are comparable with and in some cases are better than those of the other methods. The extraction time in this method is <5 min, which was shorter than that reported in Table 7. In addition, this method is very simple, easy to use, inexpensive, and environmentally benign.

Conclusions

DLLME combined with UV-Vis spectrophotometry provides an available, rapid, easy-to-operate, eco-friendly, and economic approach in chemical analysis. The potential of DLLME-UV-Vis for extraction, preconcentration, and quantification of Pb in urine samples of cyclists has been clearly demonstrated. Chemometrics was used to optimize the independent variables, including: type and volume of extracting and dispersing solvents, pH, ionic strength, and chelating agent concentration. The results of validation study illustrated the proper sensitivity, specificity, precision, selectivity, and ruggedness of the proposed method for assessing Pb in different urine samples.

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Compliance with Ethical Standards All the participants were informed about the purpose of the study and gave informed consent. Experimental procedures were approved by the Ethics Committee of Islamic Azad University, Varamin-Pishva Branch.

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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