



Application of ultrasonically modified cloud point extraction method for simultaneous enrichment of cadmium and lead in sera of different types of gallstone patients



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ABSTRACT

A novel and greener ultrasonically assisted/modified cloud point extraction procedure for the simultaneous preconcentration of lead and cadmium in serum samples of different types of gallstone patients was developed. The chelates of the under study metals, formerly formed with 8-hydroxyquinoline, were extracted in the micelles of a nonionic surfactant prior to analysis by flame atomic absorption spectrometer (FAAS). After the arrival of the cloud point, the critical micellar mass produced was homogeneously dispersed in the aqueous phase with the help of ultrasound energy. The reliability of the developed procedure was tested by relative standard deviation (% RSD), which was found to be < 5%. The performance of the proposed procedure was checked by applying to certified reference material and spiking standard in real samples. All the experimental parameters were optimized. The developed procedure of Um-CPE was applied successfully for the analysis of the target heavy metals in serum samples of different types of gallstone patients and referents. The higher levels of the understudy metals were observed in the patients as compared to the referents but the levels of the both metal were found to be considerably higher in patients with pigmented gallstones.

1. Introduction

Gallstone (GS) illness is intended to be an imminent health problem throughout the world affecting millions of the people [1–4]. Most of the time GS disease remain silent but still it is considered to be the main source of the huge health care cost and sometime its complications may result into life threatening events. According to the statistics, 49,000 and 700,000 cholecystectomies are performed annually in the United Kingdom and United States respectively, where the treatment cost for these patients account \$6.2 billion [5,6]. A recent survey based on the data collected from Hospital Episode Statistics for admissions from the Department of Health in England have indicated an increase of 30% and 64% for GS incidences in male and female respectively [7].

GS have three kinds on the basis of their major chemical constituents i.e. Cholesterol, mixed and pigmented. Cholesterol GS contain cholesterol as their main constituent, pigmented GS have higher amount of bilirubin and bilirubinate while mixed GS are the combina-

tion of the other two [8]. It is shown that some minor and trace elements may play an important role in the genesis of GS. The association of minor and trace elements concentration with various health conditions and GS formation are of prime interest in the field of medicine from very long time. On the other hand it is an accomplished fact that toxic elements can impart its harmful effect even at very low level [9]. It is reported many times in the literature that heavy metals like Cd and Pb have strong relations with the process of carcinogenicity [10,11]. The elevated levels and accumulation of the carcinogenic metals in the human body including hepatobiliary system and GS, come through various negative environmental activities like pollution, tobacco smoke and contaminated diet [12,13].

Several instrumental methodologies like electro analytical, electro thermal and flame atomic absorption spectroscopy, inductive coupled plasma joined with optical mass and emission spectrometry have been in practice for the determination and quantification of heavy metal ions in various biological and environmental matrices [14–19]. The use of

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flame atomic absorption spectroscopy (FAAS) among the mentioned techniques for the analysis of metals is very much popular due to its ability to detect and quantify very low level of metals as well as have an advantage of being simple and low cost apparatus [20–22].

Most of the time analysis of toxic elements in different types of organic and inorganic matrices may be a tough analytical assignment due to its extremely low level and multifarious arrangement in sample matrices, in this regard the analysis of such metals may be carried out either with the help of an advanced instrumental techniques or enhancing the capabilities of the available instrumental technique by bringing the analyte concentration to the promising range of the instrument in use with the help of a pre-concentration method. For the enrichment of heavy metals a number of analytical procedures have been reported in the literature like, liquid–liquid, solid phase and cloud point extraction methods [22–26]. A simple flame atomic absorption spectrometer has been shown with an enhance efficiency in term of detection limit, calibration, accuracy and results of the method by coupling with a preconcentration step before the analysis [27].

Cloud point extraction (CPE) has been shown to be an established greener procedure for the enrichment of heavy metals in several media [28]. CPE procedure is based on the phase separation behavior of the non-ionic surfactants in aqueous solutions after attaining a particular temperature i.e. cloud point and thus capturing the analytes in the form of various metal complexes. The existence of metal complexes in non-ionic surfactant rich phase is due to the pure hydrophobic interaction between the metal chelates and surfactant.

Dispersions of the micelle produced in the solution after the arrival of cloud point is an important parameter which have a strong influence on the extraction efficiency as well as on the analysis time [29,30]. Energy must be needed for the dispersion of the micelles in the solution which could be applied by several means such as mechanical agitation (valve homogenizer, stirrer, mixer, colloid mill) and ultrasound generation [31]. Nowadays ultrasound energy have gain an immense popularity to be used as a mean of safe energy to disperse the solutions due to its low cost and greener nature [32–34]. Sonication increases the interaction between the nonionic surfactant and the analyte in the solution in a short time reducing the analysis time.

Ultrasound energy produces cavitation effect in solution which enhances the mass transfer and the contact area between two media, while propagating through the solution due to the physical phenomena like micro-turbulence, micro-streaming, acoustic (or shock) waves and micro-jets [35]. The advantages of using ultrasound energy includes, reduced concentration gradients, more operative mixing and micro-mixing, rapid mass transfer, faster start-up and thus enhancing the preconcentration efficiency of the method by decreasing both the external and internal resistance to transport of analyte [36,37].

The aim of this study is to introduce an ultrasonically modified cloud point extraction method (*Um*-CPE) for the preconcentration of Cd and Pb in sera of different types of GS patients and referents. In present work the efficiency of the developed method in term of, limit of quantification, limit of detection, linear dynamic range, analyte recovery, reduced analysis time, low cost, safety and greener approach have been enhanced with the aid of ultrasound energy. 8-hydroxyquinoline (8-HQ) was used as a complexing agent for the retention of the target metals, the metal chelates formed are then extracted with the help of non-ionic surfactant known as (TX-114). The preconcentration efficiency of the developed method (*Um*-CPE) largely depends on several related parameters, which were carefully identified and optimized. The optimization of such parameters enhances the enrichment efficacy of the understudy metals in acid digested sera samples of different types of GS patients and healthy referents, collected at Mardan medical complex and teaching hospital Mardan, Pakistan. The method, we are proposing, has not been reported elsewhere as per our knowledge.

2. Experimental

2.1. Reagents and chemicals

All acids and chemicals were of analytical grad supplied by Merck (Darmstadt, Germany). For experimental work ultrapure water prepared by ELGA Lab Water system (Bucks, UK) was used. To prepare 0.6% (v/v) solution of non-ionic surfactant polyethyleneglycol *tert*-octylphenylether (Triton X-114) was attained from Sigma (St. Louis, MO, USA). Certified standard solutions (1000 µg/ml; Fluka kamika, Switzerland) of Cd and Pb were diluted to make working solutions before analysis. 100 mL ethanol (Sigma) was taken to solubilize 0.5 g of 8-hydroxyquinoline (8-HQ) (Fluka) for the preparation of 0.5% solution of the reagent. The pH of the solutions was adjusted with the help of acetate and phosphate buffers (Merck). Certified reference material for serum Clincheck Control lyophilized * human serum Recipe (Munich, Germany) was employed. In order to avoid contamination, acid-washed glass wares rinsed with ultrapure water were used for the storage and preparation of reagents and standards solutions.

2.2. Instrumentation

Microwave digestion of all the samples was carried out by a PEL (PMO23 Osaka, Japan) domestic microwave oven (maximum heating power of 900 W). Thorough mixing of solutions was carried out by means of a programmable ultrasonic water bath, model no: SC-121TH (Sonicor, Deep Park, NY, USA), with an intensification frequency of 35 kHz and temperature range of 0–80 °C. The working conditions were set according to the manufacturer recommendation and as stated in our previous Study [38]. A pH meter (Ecoscan Ion 6, Malaysia) was used to measure the pH. The phase separation was assisted with WIROWKA Laboratory jna type WE-1, nr-6933 centrifuge machine (speed range 0–6000 rpm, timer 0–60 min, 220/50 Hz, Mechanika Pheczyjna, Poland). A Perkin Elmer (Norwalk, CT, USA) A Analyst 700 atomic absorption spectrometer with (laminar flame burner and deuterium background corrector) was used for the determination of the elements. A hollow cathode lamp (single element) was set at the current of 7.0 mA and spectral bandwidth of 0.7 nm. The wavelengths for analysis of cadmium (Cd) and lead (Pb) were set at 228.8 and 283.3 nm, respectively. The burner height and acetylene and air flow rates were adjusted to attain the maximum absorbance signal.

2.3. Biological sample collection protocol and pre-treatment

The blood (serum) samples of (n = 172) patients having different types of gallstone of age group 20–55 years, admitted in surgical unite of Mardan medical complex and teaching hospital Mardan, Pakistan for performing their cholecystectomy were collected. For comparative purpose the serum samples (n = 68) from healthy age and sex matched referents were also collected. Prior to the start of study, a questionnaire was developed from all patients and control referents in order to gather information about their racial origin, physical data, nutritional routines, and age. Before starting this study an approval was obtained from the ethical review committee of Mardan medical complex and teaching hospital Mardan, Pakistan and University of Sindh, Jamshoro. All patients and control referents were provided with a written consent before the collection of their samples, confirming that they were willing for giving their blood samples and were informed about the complete experimental procedure. The demographical data of gallstone patients and control referents were described in Table 1.

The venous blood samples (5 ml) from referents and patients (before the induction of anesthesia) were taken out by means of syringe, and then transferred into jelly tube * tubes (Becton Dickinson, Rutherford, NJ, USA). The blood samples were made to clot at room temperature for 15–30 min. After the complete clotting, centrifuged at 2500 rpm for 5–10 min. The supernatant fluid (serum) was separated with the help of

Table 1
Demographics of different types of gallstone patients and control referents age group (20–55 years).

Male				Female			
Referents	Patients			Referents	Patients		
	Cholesterol ^a	Pigment ^a	Mixed ^a		Cholesterol ^a	Pigment ^a	Mixed ^a
33	40	8	12	35	80	12	20

^a Different types of gallstones patients.

Pasteur pipette and labeled accordingly. It was stored at $-4\text{ }^{\circ}\text{C}$ until further analysis. Furthermore the GS patients were grouped into, cholesterol, pigmented and mixed type GS patients with the help of visual aid and FTIR analysis of the stone recovered from them during their cholecystectomies.

2.4. Microwave-assisted acid digestion

Triplicate samples of each serum sample (0.5 ml) of both referents and patients were taken into PTFE flasks. 2 ml of concentrated HNO_3 and H_2O_2 in ratio of 2:1 (v/v) were added. The flasks were kept at room temperature for 10 min. The contents were then subjected to microwave heating for 5–10 min, detail procedure is reported in previous work [38,39]. The clear solution after digestion was made up to 25 ml in volumetric flask with 0.2 N HNO_3 . Blanks were also prepared simultaneously. The validity and accuracy of methodology was checked by spiking standards in real samples at three levels (Table 2).

2.5. Procedure for Um-CPE

For Um-CPE method, triplicate of acid digested serum samples (25 mL) of each patient and referents, six replicate of standard solutions (10 mL) containing Cd and Pb (in the range of $1.5\text{--}200\text{ }\mu\text{g L}^{-1}$) were taken separately in centrifuge tubes having glass stopper (50 mL in capacity). Then chelating agent and nonionic surfactant in the range of (0.1–0.4%, w/v and 0.1–0.4%, v/v) were added to the tube respectively. The pH of the tube solution were kept in the range of 4–10 by adding buffers. For the arrival of the cloud point the tubes were kept in an ultrasonic water bath in the temperature range of $35\text{--}60\text{ }^{\circ}\text{C}$ for a time period of 5–20 min. The micelles formed in the solution after the arrival of cloud point were dispersed with the help of ultrasound energy for a time period of (0.25–03) min. Further the surfactant rich phase was separated from the aqueous solution through centrifugation at

3500 rpm for 5–10 min. An ice bath treatment of the tubes were carried out for 5 min in order to collect the surfactant rich phase in much more viscous form. Then the removal of the aqueous phase was carried out in a gentle manner with the help of a syringe. The viscous surfactant rich phase was made diluted with the help of ethanolic solution of nitric acid then subjected to the FAAS for the analysis of the target metals (Fig. 6).

3. Results and discussion

The efficiency and selectivity of the developed method (Um-CPE) for the enrichment of the understudy elements largely depends on the experimental parameter like, amount of nonionic surfactant and chelating agent, pH, temperature, equilibration time and dispersion of the micelle formed. The effect of all these parameters on the proposed methods were carefully examined and optimized by applying the given procedure of the Um-CPE to six replicates of certified standard solution ($10\text{ }\mu\text{g L}^{-1}$) of Cd and Pb.

3.1. Effect of pH

pH is known to have a pronounced effect on the efficiency of the Um-CPE method. It performs a crucial role in the formation and stability of metal chelates, thus affecting the percent recovery of the understudy metals. Metal chelation dependence on pH is related to both the metal chemistry in the solution and the ionization state of the complexing agent which affects the availability of binding sites [40]. For this purpose the % recovery of the understudy metals in certified standard solution ($10\text{ }\mu\text{g L}^{-1}$) was checked in the pH range of 4–10, using acetate and phosphate buffer, while the desired pH adjustment was carried out with the addition of 0.1 mol L^{-1} HCl/NaOH. The maximum recovery of the target analytes was obtained in the pH range between 6 and 8 as shown in Fig. 1. pH 7 was chosen as the optimum pH and rest of the experiments were carried out at pH 7. The ligand we

Table 2
Determination of Cd and Pb ($\mu\text{g L}^{-1}$) in certified reference material of serum (ClinCheck control-lyophilized[®] serum) and spike recovery study in real serum samples of gallstone patients (n = 6)^a

Metals	Certified values	Obtained value		% Recovery ^b	
		Without Pre-concentration	With Pre-concentration	Without Pre-concentration	With Pre-concentration
Cd	4.60 ± 1.20	4.43 ± 0.500	4.54 ± 0.390	96.3	98.6
Pb	–	–	–	–	–
<i>Spike recovery study</i>					
Added	Found (Mean \pm SD)			% Recovery ^c	
	Cd	Pb	Cd	Pb	
0	4.91 ± 0.430	52.1 ± 3.79	–	–	
5	9.86 ± 0.570	56.9 ± 3.83	99.0	97.0	
10	14.8 ± 0.540	61.9 ± 3.91	98.9	98.0	
15	19.7 ± 0.690	66.8 ± 3.96	98.6	98.3	

Keys:

^a Mean \pm Standard deviation.

^b %Recovery = $\frac{\text{Obtained value}}{\text{Certified value}} \times 100$

^c %Recovery = $\frac{\text{Certified value} + \text{spike addition}}{\text{added}} \times 100$

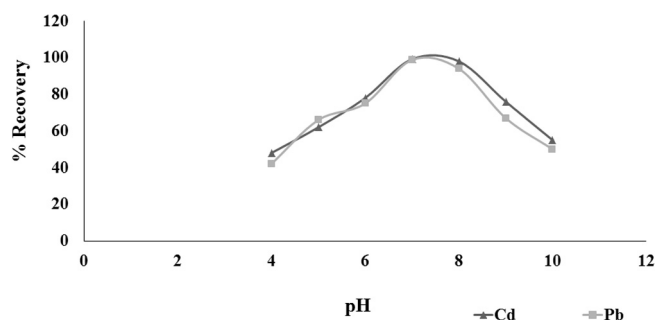


Fig. 1. Influence of pH on the percent recovery of cadmium (Cd) and lead (Pb). Conditions: $10 \mu\text{g L}^{-1}$ cadmium (Cd) and lead (Pb) and 0.3% (w/v) 8-HQ and 0.2% (v/v) Triton X-114. Other experimental conditions are explained under procedures.

are using complexes the metal ions at neutral pH because at acidic pH protonation of the ligand takes place and at basic pH hydroxide of the metals form and no ligand and metal ions are available for complexation respectively. [40].

3.2. Effect of Triton X-114 concentration

The selection of an appropriate nonionic surfactant and its effective concentration to accumulate metal chelates is an important step in the preconcentration procedure for the target analyte. Separation of metal ions by extraction method involves the prior formation of a complex with sufficient hydrophobicity to be extracted in a small volume of surfactant-rich phase. The temperature corresponding to the cloud point is correlated with the hydrophilic property of surfactants, thus an appropriate surfactant is important. Surfactants that have too high or too low cloud point temperatures are not suitable for CPE of trace metals [41]. The present study was carried out with Triton X-114 as a nonionic surfactant offering the advantages of, its ability to form two phases at low temperature (23–26 °C) and can easily settle down in the aqueous phase due to its higher density, after centrifugation [42]. The concentrating efficiency of the Triton X-114 to accumulate the metal chelate was checked in term of the % recovery of the understudy metals. Triton X-114 was employed in the concentration range of (0.1–0.4%, v/v) (Fig. 2), and maximum % recovery was observed at the concentration ranges between 0.2 and 0.3%. For further study the concentration of 0.2% was selected as optimum because higher concentration of the nonionic surfactant may weaken the analyte signal.

3.3. Effect of chelating agent concentration

8-hydroxyquinoline is a non-polar chelating agent that can readily form chelates with target metals both in certified standard and real samples [44,45]. In the present study the complexation of the selected metals were carried out with 8-hydroxyquinoline in the concentration

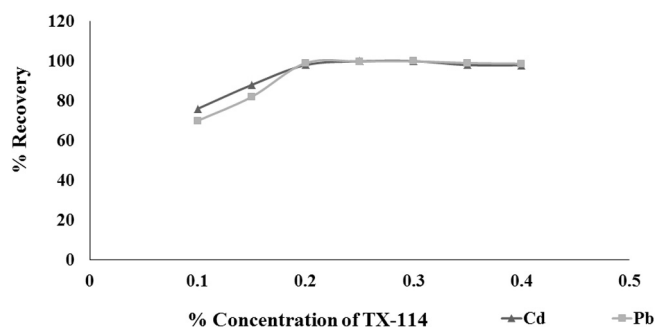


Fig. 2. Variation of the percent recovery of cadmium (Cd) and lead (Pb) as a function of Triton X-114 concentration. Conditions: $10 \mu\text{g L}^{-1}$ cadmium (Cd) and lead (Pb), 0.3% (w/v) 8-HQ and pH 7. Other experimental conditions are described under procedures.

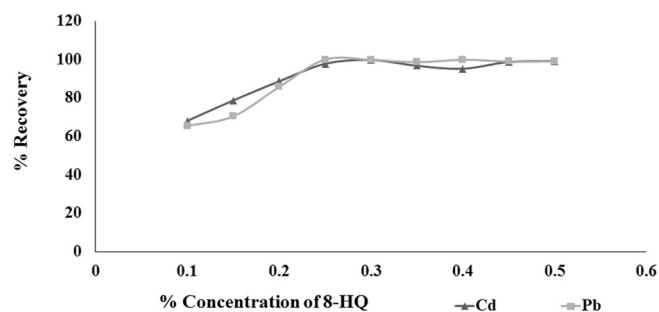


Fig. 3. Influence of the 8-hydroxyquinoline concentration on the percent recovery of cadmium (Cd) and lead (Pb). Conditions: $10 \mu\text{g L}^{-1}$ cadmium (Cd) and lead (Pb), 0.2% (v/v) Triton X-114 and pH 7. Other experimental conditions are described under procedures.

range of 0.1–0.5% as shown in the Fig. 3. Maximum % recovery of the analytes were noticed at 0.3% concentration of the ligand, no effect on the % recovery was observed for further increase in the concentration. Slight reduction of % recovery at high concentration of 8-HQ was observed, this is because the excess of non-ionic 8-HQ molecules are presumably trapped in micelles [41]. Hence 0.3% was selected as an optimum ligand concentration for rest of the work.

3.4. Effect of dispersion mood and time

It is well known that the dispersion of the contents of the solution enhances the interaction between analyte, chelating agent and micelles which improves the analyte entrapment by the complexing agent and mass transfer into the surfactant rich organic phase, which are further separated by centrifugation. It is expected that shaking the tube after the addition of the extraction mixture solvent and before centrifugation, would strongly influence the extraction efficiency and recovery of the proposed method, because it facilitate a more intimate and prolonged contact between analyte and extraction mixture in aqueous and organic phases. Various strategies for assisting dispersion have been studied, which include manual shaking, vortex and ultrasound energy as shown in the Fig. 4. Among these strategies the dispersion of the extraction mixture with the help of ultrasound was found to be best in term of the extraction efficiency and recovery of the analyte, reduce analysis time, less energy consumption, low chances for reagents losses, greener approach and low cost. The sonication time was studied for the proposed method for the time period of (0.25–03) min. The % recoveries of the understudy metals increases with an increase in the sonication time as shown in the Fig. 5. The % recoveries reaches its maximum value for sonication the solution up to 1 min, further increase in sonication time have no effect on the % recoveries of the metals. Sonication for 1 min was selected as optimum time and was implied to rest of the experiments. For vortex assisted dispersion the vortex mixing time was studied in the range of (1–6) min. The optimum vortex time for maximum % recoveries was found to be 3 min, while the % recoveries for the understudy metals were found to be ~92–93%. In case of manual shaking, the contents of the solution was disperse with the help of hand shaking for 6 min and the % recoveries for the understudy elements were found to be ~80%.

3.5. Effects of incubation temperature and time

The incubation temperature for the Triton X-114 has a strong influence on the performance of CPE. A better equilibration temperature above the cloud point of the Triton X-114 play a crucial role in the efficient separation of the phases and extraction of the analyte. For Triton X-114, an increase in the cloud point temperature also leads to a slight decrease in the volume of the surfactant-rich phase. This can be interpreted that as the temperature increases, the hydrogen bonds are disrupted and dehydration occurs. It is desirable to use the shortest



Fig. 4. Various dispersion schemes for the dispersion of extraction mixture.

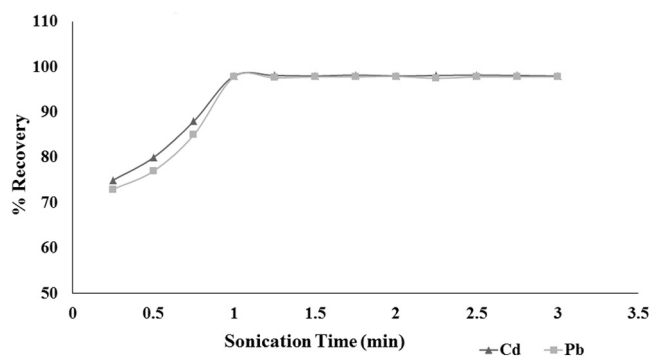


Fig. 5. Effect of sonication time on the percent recovery of cadmium (Cd) and lead (Pb). Conditions: $10 \mu\text{g L}^{-1}$ cadmium (Cd) and lead (Pb), 0.2% (v/v) Triton X-114, 0.3% (w/v) 8-HQ and pH 7. Other experimental conditions are described under procedures.

incubation time and the lowest possible incubation temperature as a compromise between completion of extraction and efficient separation of the phases [41]. In present study the effect of the equilibrium temperature on % recovery of the understudy metals were carried out in ultrasonic bath at temperature range of 35–60 °C. Maximum recovery was observed at 45 °C. The effect of the equilibration time on efficiency of the proposed method was also explored. For this purpose the incubation time was studied between 5 and 20 min, 10 min of equilibration was sufficient for the maximum recovery of the target analytes.

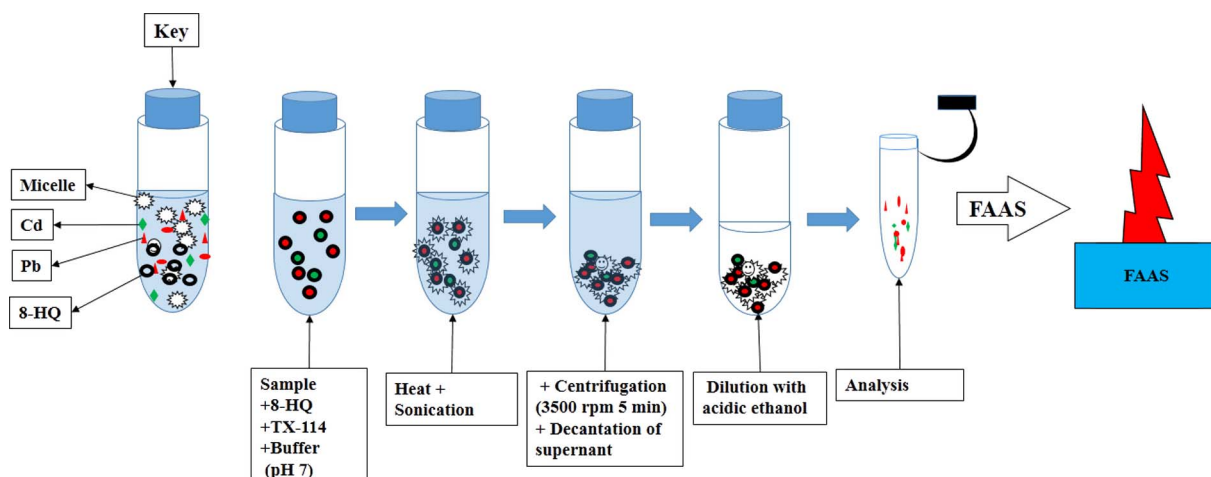


Fig. 6. Graphical representation of Um-CPE procedure.

3.6. Interferences

A number of cations other than the target analytes can be chelated by the ligand, which may alter the extraction capability of the understudy metals. To investigate the effect of foreign ion on efficiency of the proposed procedure, a 10 ml solution comprises of the certified standards of the target analytes ($10 \mu\text{g L}^{-1}$) and foreign ions in different ratios were checked by applying the developed procedure to the test solution. It is shown in the Table 3 that the added foreign ions were well tolerated with in the tolerance limit (less than 5% error). Further the use of 8-hydroxyquinoline in a relatively higher concentration is advisable for the complete complexation of the target metals even in the presence of the foreign ions.

3.7. Analytical figures of merit

The linear dynamic range of the proposed method for Cd and Pb were found to be in the concentration range of 1.5 to $200 \mu\text{g L}^{-1}$ at optimal parameters. The characteristics analytical performance of the developed procedure in term of, linear range, quantification limit (LOQ), detection limit (LOD), preconcentration factor (PF), enhancement factor (EF) and percent relative standard deviation (% RSDs) are given in the Table 4. The enhancement factor (EF) and preconcentration factor (PF) were found to be 31 and 62.5 respectively. The enhancement factor was calculated as the ratio between slopes of the calibration graphs with and without preconcentration. The preconcentration

Table 3
Effect of some foreign ions on the % recoveries of Cd and Pb determined *Um*-CPE ($\mu\text{g/L}$).

Ion	Conc: of interfering ions	% Recovery
Na	> 10,000	99.5
K	> 10,000	100
Ca	> 10,000	99.1
Mg	> 10,000	98.1
Al	~100	98.8
Fe	~100	97.5
Mn	~100	98.8
Co	50	99.5
Ni	50	100
Zn	50	100
Cr	50	99.1

Table 4
Analytical figure of merits for the developed *Um*-CPE method for the preconcentration of Cd and Pb ($\mu\text{g L}^{-1}$) in serum samples of different types of gallstone patients.

Parameter	Value	
Elements	Cd	Pb
Range	1.50–200	1.50–200
R ²	0.999	0.997
EF ^a	30.5	30.3
PF ^b	62.5	62.5
% RSD ^c	3.56	4.12
LOD ^d	0.052	0.450
LOQ ^e	1.62	1.80

Keys:

^a Enhancement Factor. Calculated as the ratio of slopes of the calibration graphs of preconcentrated standards to that obtained without preconcentration.

^b Calculated as the ratio between sample volumes after and before preconcentration. $PF = \frac{V_f}{V_i}$, where V_f and V_i are the sample volumes after and before preconcentration respectively.

^c % Relative standard deviation ($n = 6$; $10 \mu\text{g L}^{-1}$) $RSD = \frac{s}{\bar{x}}$, where s is standard deviation and \bar{x} is mean of six replicate samples of $10 \mu\text{g L}^{-1}$.

^d Limit of Detection. Calculated as three times the ratio between standard deviation of 10 blank signals and slope of the calibration graph $LOD = \frac{3s}{m}$, where s is standard deviation of 10 blank readings and m is slope of the calibration graph.

^e Limit of Quantification. Calculated as ten times the ratio between standard deviation of 10 blank signals and slope of the calibration graph $LOQ = \frac{10s}{m}$, where s is standard deviation of 10 blank readings and m is slope of the calibration graph.

Table 5
Comparative data of analytical characteristics of *Um*-CPE for Cd and Pb with previously reported pre-concentration methods.

Sample	Metal	Method	Technique	EF ^a	PF ^b	LOD $\mu\text{g L}^{-1}$	Range $\mu\text{g L}^{-1}$	Ref.
Drinking water	Cd	^c CPE	FAAS	–	80.0	0.220	5.00–10.0	[43]
Human hair	Cd	CPE	FAAS	–	22.0	0.620	3.00–300	[44]
Water	Cd	^d MS-CPE	FAAS	54.0	–	0.290	5.00–100	[45]
Water	Pb	^e SPE	FAAS	–	100	0.510	–	[46]
Blood	Pb	CPE	FAAS	56.0	–	1.14	–	[47]
Food	Pb	^f NL-CPE	FAAS	30.0	–	1.90	3.40–130	[48]
fish muscle	Cd	^g m-CPE	FAAS	78.9	25.0	0.0800	2.00–50.0	[49]
fish muscle	Pb	<i>m</i> -CPE	FAAS	84.2	25.0	0.560	2.00–50.0	–
Lake water	Cd	<i>m</i> -CPE	FAAS	78.9	62.5	0.0800	2.00–50.0	–
Lake water	Pb	<i>m</i> -CPE	FAAS	84.2	62.5	0.560	2.00–50.0	–
Waste water	Pb	SPE	FAAS	–	100	4.50	10.0–200	[50]
water	Cd	CPE	FAAS	57.5	–	0.099	0.099–50.0	[51]
	Pb	CPE	FAAS	55.6	–	1.10	1.10–160	–
Serum	Cd	^h <i>Um</i> -CPE	FAAS	30.5	62.5	0.052	1.5–200	Present work
Serum	Pb	<i>Um</i> -CPE	FAAS	30.3	62.5	0.450	1.5–200	–

Keys:

^a Enhancement factor.

^b Preconcentration factor.

^c Cloud point extraction.

^d Multistep cloud point extraction.

^e Solid phase extraction.

^f Non-ligand dual cloud point extraction.

^g Modified cloud point extraction.

^h Ultrasonically modified cloud point extraction.

tration factor (PF) can be attributed to the ratio of volume of the initial solution to that of the final solution after preconcentration. An effective preconcentration method should increase the extracting capability by increasing the phase volume ratio (V_i/V_f), where V_i and V_f are volumes of sample before and after preconcentration. Among other variables, the PF depends on the phase relationship, the distribution constant of the analytes between the phases, and concentration of the surfactant used [41,43]. The detection limit (LOD) and quantification limit (LOQ) were obtained as 3 and 10 times, respectively, the ratio between the standard deviation of 10 readings of blank and slope of the calibration graphs [41,43]. The obtained LOD was reasonably low and reliable for the determination of extremely low level of the understudy metals in the serum samples. Certified reference material for serum (Clincheck Control lyophilized[®] human serum Recipe) was studied using the developed procedure in order to check the precision and accuracy of the method, standards of the understudy metals were also spiked into the real samples for further authenticity of our results (Table 2). Introduction of ultrasonic energy has boosted up the limit of quantification, limit of detection, linear dynamic range, analyte recovery and reduced time and cost of analysis. Moreover it is safe and greener approach as compared to simple cloud point extraction because the use of ultrasound energy is environmental friendly and mechanical loss of the reagents is minimum.

3.8. Comparison with other preconcentration methods

The analytical performance of the developed procedure using 8-hydroxyquinoline as a chelating agent for the analysis of the understudy metals in sera of different types of GS patients and healthy referents were compared to previously reported preconcentration methods as shown in Table 5, [41,43,46–52]. The comparison table reveals that our developed procedure is superior in term of linear range, detection limit (LOD), preconcentration factor (PF) and enhancement factor (EF) except in few cases. The improved analytical figures of merit of *Um*-CPE make it to be used an alternative preconcentration method for the understudy metals in biological matrices.

3.9. Application to real samples

Um-CPE was successfully applied for the analysis of Cd and Pb in

Table 6
Concentration of Cd and Pb ($\mu\text{g L}^{-1}$) in serum samples of different types of gallstone patients of both genders.

Metals	Gender	Referents	Cholesterol	Pigment	Mixed
Cd	Male	2.27 ± 0.19	4.91 ± 0.43	9.04 ± 0.34	7.32 ± 0.43
	Female	2.17 ± 0.13	4.71 ± 0.56	8.86 ± 0.49	7.09 ± 0.58
Pb	Male	24.2 ± 2.22	52.1 ± 3.79	128 ± 10.4	83.2 ± 6.39
	Female	21.8 ± 1.99	49.2 ± 3.84	116 ± 9.48	77.2 ± 5.79

blood serum of patients having different types of GS and healthy age and sex matched referents. The mean concentration of Cd and Pb along with standard deviation in serum samples of both male and female referents subjects and gallstone patients are given in the Table 6. The contents of Cd in serum samples of male referent subjects and GS patients having different types of stones i.e. (cholesterol, pigmented, mixed) of age group 20–55 years were found at 95% confidence interval (CI: 2.20–2.30) $\mu\text{g L}^{-1}$ and (CI: 4.80–5.00), (CI: 8.80–9.28), (CI: 7.10–7.50) $\mu\text{g L}^{-1}$ respectively. While in case of female referents subjects and GS patients (cholesterol, pigmented, mixed) have serum Cd levels (CI: 2.15–2.20) $\mu\text{g L}^{-1}$ and (CI: 4.60–4.80), (CI: 8.60–9.10), (CI: 6.85–7.35) $\mu\text{g L}^{-1}$, respectively. The range of Pb level in serum samples of male and female referent subjects were found at 95% confidence interval (CI: 23.5–24.8) and (CI: 21.4–22.3) $\mu\text{g L}^{-1}$ respectively. While serum Pb contents in male and female GS patients with different types of stones i.e. cholesterol, pigmented, mixed were found at 95% confidence interval (CI: 51–53.3), (CI: 122–134), (CI: 80.4–86.0) and (CI: 48.4–50.0), (CI: 112–120), (CI: 75.0–79.5) $\mu\text{g L}^{-1}$ respectively. The resulted data indicate that the levels of the both metals were elevated in the serum of patients as compared to that of the referents as shown in the Table 6. The sera of all types of GS patients contain higher concentration of the understudy metals but the difference was significant ($p < 0.005$) for the patients with pigmented type GS as compared to the referents. The concentration of Cd and Pb in different types GS patients were found in the decreasing order, Pigmented > mixed > cholesterol.

Higher levels of Pb in livers induce the generation of free radicals, but its low level may also affect the normal biochemical process in the hepatobiliary system and Pb may precipitate into GS [53,54]. Cadmium and lead reduce the production of N-oxide in gallbladder which is important for the maintenance of normal tone of gallbladder and promote hydroxyl radical formation, each of these effect facilitate the process of stone formation in gallbladder [55,56].

4. Conclusion

The *Um*-CPE has proved to be a promising procedure for analysis of the target analytes when joined with FAAS in the acid decomposed sera of different types of GS patients and healthy referents. Application of ultrasound energy in the proposed method has largely enhanced its efficiency, the dispersion of the micelle formed after arrival of the cloud point with ultrasound energy increases the interaction between micelle and metal chelates in shorter time and also increases the contact area between the two media (surfactant enriched and aqueous medium) which facilitate the mass transfer of the analytes from the aqueous phase to the micelles enriched phase which enhances the extraction capability of the developed procedure to a greater extent. The proposed method of *Um*-CPE has an enhanced analytical performance as compared to other preconcentration methods used for the enrichment of Cd and Pb in various matrices reported in the literature. It is safe and greener approach as compared to simple cloud point extraction because the use of ultrasound energy is environmental friendly and mechanical loss of the reagents is minimum. The authenticity of the developed procedure was checked by examining the serum (Clincheck Control lyophilized * human serum Recipe) and standard addition to the real

samples. The developed procedure is advantageous in terms of, greener nature, safety, simplicity, low cost, reproducibility, LOQ, LOD, higher extraction efficiency, linear range and the utilization of FAAS which is a routine instrument in most laboratories. The developed procedure can be used for the determination of the target analytes with good accuracy and reproducibility in the samples under study. The average concentration of both metals were found higher in serum samples of the patients having different types of GS as compared to the serum samples of healthy and age matched referents. Among different types of GS patients the serum of pigmented GS patients contains significantly higher concentration of both metals ($P < 0.05$).

Both the metals play an important role in the chemistry of liver and gallbladder. Liver with high levels of Pb and Cd may suffer from free radical generation and disturbed biochemical process in the hepatobiliary system. Elevated concentrations of these metals lessen the production of nitrogen oxides in gallbladder. As a consequence the process of stone formation in gallbladder is facilitated.

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Conflict of interest

All authors declare no conflict of interest.

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