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Spectrochimica Acta Part B 62 (2007) 1019 – 1027

SPECTROCHIMICA ACTA PART B

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Cloud point extraction for the determination of lead and cadmium in urine by graphite furnace atomic absorption spectrometry with multivariate optimization using Box–Behnken design ☆

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Received 8 January 2007; accepted 31 May 2007
Available online 7 June 2007

Abstract

Cloud point extraction (CPE) is proposed as a pre-concentration procedure for the determination of Pb and Cd in undigested urine by graphite furnace atomic absorption spectrometry (GF AAS). Aliquots of 0.5 mL urine were acidified with HCl and the chelating agent ammonium *O*,*O*-diethyl dithiophosphate (DDTP) was added along with the non-ionic surfactant Triton X-114 at the optimized concentrations. Phase separation was achieved by heating the mixture to 50 °C for 15 min. The surfactant-rich phase was analyzed by GF AAS, employing the optimized pyrolysis temperatures of 900 °C for Pb and 800 °C for Cd, using a graphite tube with a platform treated with 500 μg Ru as permanent modifier. The reagent concentrations for CPE (HCl, DDTP and Triton X-114) were optimized using a Box–Behnken design. The response surfaces and the optimum values were very similar for aqueous solutions and for the urine samples, demonstrating that aqueous standards submitted to CPE could be used for calibration. Detection limits of 40 and 2 ng L^{-1} for Pb and Cd, respectively, were obtained along with an enhancement factor of 16 for both analytes. Three control urine samples were analyzed using this approach, and good agreement was obtained at a 95% statistical confidence level between the certified and determined values. Five real samples have also been analyzed before and after spiking with Pb and Cd, resulting in recoveries ranging from 97 to 118%.

Keywords: Cloud point extraction; Graphite furnace atomic absorption spectrometry; Urine; Cadmium determination; Lead determination; Permanent modifier

1. Introduction

Monitoring the presence of toxic trace elements in biological fluids is an extremely important task to evaluate occupational and environmental exposure. Cadmium and lead are frequently referred to as hazardous elements. Elevated Cd concentrations in urine represent long-term exposure and significant accumulation, particularly in the kidneys and liver [1], while elevated Pb concentration in urine is associated with recently absorbed amounts [2].

Monitoring trace element concentrations in biological materials, particularly biological fluids, might be considered a difficult analytical task, mostly due to the complexity of the matrix and the low concentration of these elements, which requires sensitive instrumental techniques and often a pre-concentration step. The traditional liquid–liquid extraction and other conventional separation methods are time-consuming and labor-intensive approaches, besides requiring relatively large amounts of high-purity and frequently toxic solvents, which have to be disposed off properly. Cloud point extraction (CPE) is based on the phase behavior of non-ionic surfactants in aqueous solutions, which exhibit phase separation after an increase in temperature or the addition of a salting-out agent

☆ This paper was presented at the 9th Rio Symposium on Atomic Spectrometry, held in Barquisimeto, Venezuela, 5–10 November 2006, and is published in the special issue of Spectrochimica Acta Part B, dedicated to that conference.

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doi:10.1016/j.sab.2007.05.008

[3,4]. CPE might be an interesting and efficient alternative, once it eliminates or reduces consumption of organic solvents significantly. Trace elements can be extracted to the surfactant-rich phase usually after formation of a hydrophobic complex with an appropriate chelating agent [5]. This approach has been successfully employed to extract and pre-concentrate several trace elements from a variety of matrices [6–9], including biological samples [10,11].

Method development for CPE requires the optimization of several experimental parameters, such as pH and the concentrations of chelating agent and surfactant. Traditional univariate optimization might be a time-consuming and labor-intensive procedure, requiring several experiments to be performed and neglecting possible interactions between variables. Multivariate optimization appears to be a more interesting and complete alternative, allowing maximum information to be obtained due to the possibility of evaluating interactions between variables. It also represents a more economical approach, as the number of experiments can be significantly reduced. One of the possibilities is to use a Box–Behnken design, in which the number of experiments (N) is defined by the equation $N=k^2+k+c_p$, where k represents the number of factors (parameters) involved in the study and c_p is the number of replicates of the central point. Box–Behnken could be seen as a cube, consisting of a central point and the middle points of the edges. However, it can also be viewed as interlocking 2^2 factorial design and a central point [12]. The use of Box–Behnken design has become of increasing interest in the field of analytical chemistry, with application to on-line pre-concentration systems [12], coal modeling [13], capillary electrophoresis [14] and gas chromatography [15], among others.

In this work, a CPE procedure using Triton X-114 as non-ionic surfactant and *O*,*O*-diethyl dithiophosphate (DDTP) as chelating agent was developed for the extraction of Cd and Pb from undigested urine samples and their determination by graphite furnace atomic absorption spectrometry (GF AAS). The optimization of the experimental variables (concentration of HCl, DDTP and Triton X-114) for the CPE will be described using a Box–Behnken design with three variables.

2. Experimental

2.1. Instrumentation

All measurements were carried out using an AAnalyst 100 atomic absorption spectrometer (Perkin Elmer, Norwalk, CT, USA), equipped with an HGA 800 longitudinally heated graphite tube atomizer and an AS-72 autosampler (Perkin Elmer). Deuterium-arc background correction was employed to correct for non-specific absorption. All measurements were performed using integrated absorbance (peak area). Hollow cathode lamps for Cd and Pb (Perkin Elmer) were operated at 4 mA and 10 mA, respectively, with a spectral bandwidth of 0.7 nm. The selected wavelengths were 228.8 nm and 283.3 nm for Cd and Pb, respectively. Aliquots of 20 μL of all samples and calibration solutions were injected directly into the graphite tube. Argon 99.996% (White Martins, São Paulo, SP, Brazil) was used as protective and purge gas. Pyrolytic graphite coated polycrystalline electrographite tubes with total pyrolytic graphite platforms (Perkin Elmer) were used throughout. The graphite furnace temperature program for the determination of both analytes is shown in Table 1.

2.2. Reagents, standards and reference materials

All chemicals were at least of analytical-reagent grade. Water was de-ionized in a Milli-Q system (Millipore, Bedford, MA, USA) to a resistivity of 18.2 MΩ cm. Hydrochloric acid (Merck, Darmstadt, Germany), methanol (Carlo Erba, Milan, Italy) and nitric acid (Carlo Erba) were further purified by double sub-boiling distillation in a quartz still (Kürner Analysentechnik, Rosenheim, Germany). Ammonium *O*,*O*-diethyl dithiophosphate (DDTP, Aldrich Chemical Co., Milwaukee, WI, USA) and octylphenoxypolyethoxyethanol (Triton X-114, Sigma, St Louis, MO, USA) were used as supplied. Stock standard solutions containing 1000 mg L^{-1} Pb and Cd were prepared by dissolution of high purity $Pb(NO_3)_2$ and CdO (SPEX, Eddison, NJ, USA), respectively, in 5% v/v HNO_3.

Three urine control samples were used for accuracy check, including Metalle U level 1 and level 2 Human Urine Control (Medichem, Steinenbronn, Germany) and Seronorm Trace Elements in Urine (Sero AS, Billingstad, Norway).

2.3. Treatment of platforms with permanent modifiers

The permanent modifier (Ru or Pd) was applied to the platform by 20 consecutive injections of 25-μL aliquots of a 1000 mg L^{-1} Ru or Pd solution into the graphite furnace; each injection was followed by a specific temperature program, as described previously [16]. The procedure resulted in the deposition of 500 μg of the modifier onto the platform surface.

2.4. CPE procedure

Urine control samples were reconstituted with exactly 5.0 mL of de-ionized water and left to stand for about 30 min. Aliquots of the reconstituted samples were directly submitted to the CPE procedure. The pre-concentration procedure started with the addition of HCl at the optimized concentrations, 0.3 mol L^{-1} for Pb or 0.5 mol L^{-1} for Cd, to aliquots containing between 25 and 100 μL of the reconstituted

Table 1
Temperature program for the determination of Cd and Pb in urine samples by GF AAS following CPE

Stage	Temperature/°C	Ramp/s	Hold/s	Ar flow rate/mL min^{-1}
Drying	80	5	10	250
Drying	110	5	10	250
Pyrolysis	800[a], 900[b]	5	25	250
Cooling	20	1	5	250
Atomization	1600[a], 1800[b]	0	5	0
Cleanout	2200	1	5	250

[a] Temperature for Cd.
[b] Temperature for Pb.

sample, depending on the analyte concentration, in 15-mL polypropylene flasks (Techno Plastic Products AG, Trasadingen, Switzerland). A stock solution containing 5% m/v DDTP was prepared in de-ionized water, and the ligand was added to the samples until the optimum concentration, 0.7% m/v for Pb or 1.00% m/v for Cd, was obtained. The optimized Triton X-114 concentration, 0.40% m/v for Pb or 0.50% m/v for Cd, was added to each mixture and the volume was completed to 14 mL with de-ionized water. The resulting solution was heated to 50 °C in a water bath for 20 min. Phase separation was accelerated by centrifuging the tubes at 3500 rpm for 20 min. The flasks were immersed in an ice bath for 10 min, allowing the elimination of the aqueous phase by simply inverting the tubes. Any residual water was removed using a Pasteur pipette. To reduce the viscosity of the surfactant phase prior to GF AAS analysis, 500 μL of methanol acidified with 0.1 mol L^{-1} HNO_3 was added to the extract. Twenty-microliter aliquots of the resulting solution were directly injected into the graphite tube by means of the autosampler and submitted to the temperature program shown in Table 1. Real urine samples obtained from non-exposed volunteers have also been analyzed without prior digestion. In this case, 500-μL aliquots have been submitted to the same CPE procedure as described above. Spike recovery tests were also carried out. In all cases, calibration was performed by submitting aqueous standard solutions of Cd and Pb to the same CPE procedure as described for the urine samples.

2.5. Optimization strategy

First, the optimization of CPE components has been evaluated by a two-level factorial design with a central point. Afterwards, the response surface methodology with the Box–Behnken design, involving the variables concentration of HCl, concentration of DDTP and concentration of Triton X-114, has been carried out. The analytical response used has been the integrated absorbance. Experimental data have been evaluated using the software Statistica 6.0. Both experimental designs have been carried out using duplicate measurements.

3. Results and discussion

3.1. Pyrolysis and atomization curves

Pyrolysis and atomization curves were established using one of the urine samples enriched with Cd (0.05 μg L^{-1}) and Pb (1 μg L^{-1}) and submitted to CPE. Aliquots containing 20 μL of the final extract in methanol were used for GF AAS analysis.

Pyrolysis temperatures below 600 °C resulted in significantly lower precision, as the matrix could not be eliminated, resulting in a high background signal that could not be efficiently corrected by the deuterium background corrector. Fig. 1a shows the pyrolysis and atomization curves for Pb in urine submitted to CPE. Three modifiers have been tested in this study: 500 μg Ru as permanent modifier, 500 μg Pd as permanent modifier [17], and the addition of 10 μg Pd in solution along with each sample injection. As can be seen in

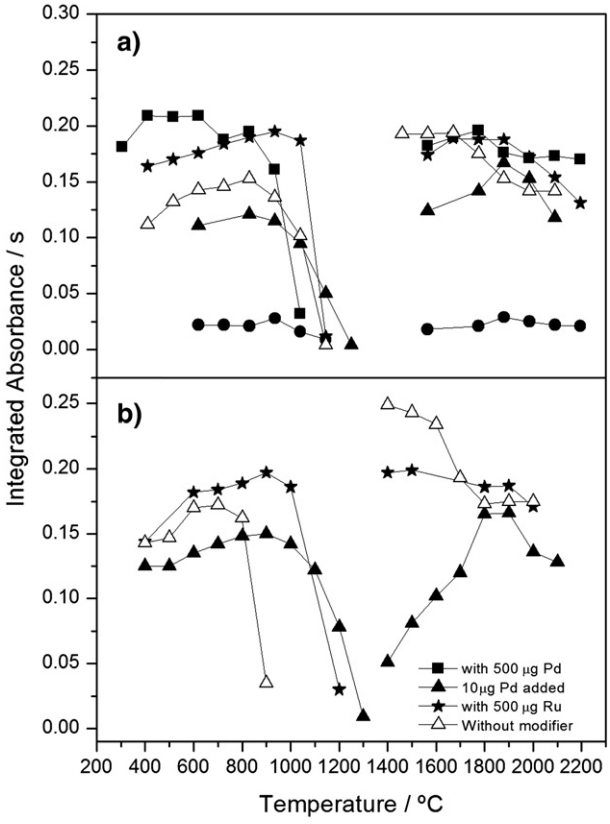

Fig. 1. Pyrolysis and atomization curves for Pb obtained after cloud point extraction from: (a) urine sample enriched with 1.0 μg L^{-1} Pb, and (b) aqueous solution containing 1.0 μg L^{-1} Pb. Atomization temperature employed for pyrolysis curves: 1800 °C; pyrolysis temperature employed for atomization curves: 800 °C.

Fig. 1a, significant thermal stabilization was achieved even without the use of a modifier, as a pyrolysis temperature of 800 °C could be used without analyte loss. This is most likely due to the presence of high levels of phosphate in urine and to the added DDTP, which can both act as chemical modifiers as reported previously [11]. The addition of Pd in solution to each sample injection resulted in the formation of a precipitate in the autosampler capillary tip, which was due to urine constituents. In addition, the use of Pd in solution did not result in significant thermal stabilization compared to the use of no modifier, as can be seen in Fig. 1a. Similarly, the use of a graphite tube treated with Pd as a permanent modifier was of no advantage either, as the thermal stability was essentially the same as without modifier. The best results were obtained using Ru as a permanent modifier, which allowed a pyrolysis temperature of up to 1000 °C to be used without analyte loss. Under these conditions, an almost background-free signal could be obtained, improving precision. An atomization temperature of 1800 °C was chosen based on the curve profile shown in Fig. 1a.

A similar evaluation was carried out using an aqueous solution submitted to CPE, and the results are shown in Fig. 1b. Although the use of Pd in solution in this case obviously did not result in the formation of precipitates, and relatively high thermal stabilization could be achieved, the use of a Ru-treated

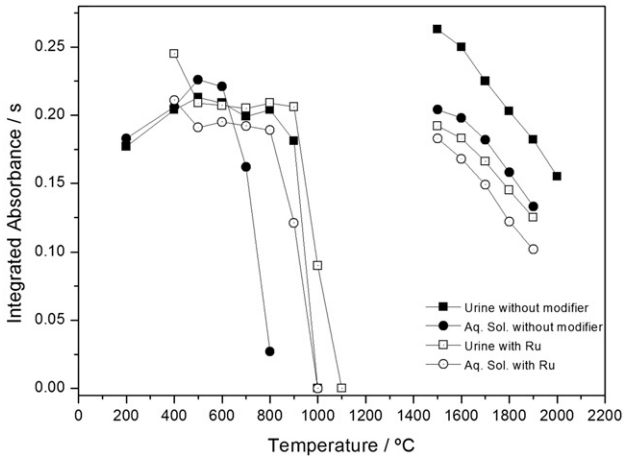

Fig. 2. Pyrolysis and atomization curves for Cd in enriched urine sample and in aqueous solution submitted to cloud point extraction (0.05 μg L^{-1} Cd). Atomization temperature employed for pyrolysis curves: 1600 °C; pyrolysis temperature employed for atomization curves: 600 °C (without modifier) or 800 °C (with Ru permanent modifier).

tube resulted in higher sensitivity and also in good thermal stabilization, similar to that observed for Pb in the urine sample submitted to CPE. Ruthenium as permanent modifier was therefore chosen for the determination of Pb in urine samples after CPE with a pyrolysis temperature of 900 °C.

Pyrolysis and atomization curves for Cd have been established in the same manner as described above for Pb, except that Ru as a permanent modifier was the only modifier tested for an aqueous solution and a urine sample submitted to CPE, as shown in Fig. 2. The use of Ru as a permanent modifier for Cd determination has already proved to be efficient in previous works [18–20]. The effect of the matrix on the thermal stabilization of Cd was particularly pronounced, as without a modifier a pyrolysis temperature of up to 900 °C could be used for urine samples, which is quite unusual for Cd. In an aqueous solution submitted to CPE, however, a significant loss of Cd was observed already at a pyrolysis temperature of 700 °C, demonstrating that the components of the urine matrix are in fact responsible for the thermal stabilization of Cd up to an additional 200 °C. DDTP is also responsible for the thermal stabilization of Cd, as in an aqueous solution significant losses can be detected already at pyrolysis temperatures above 300 °C, while for Cd submitted to CPE significant losses occur only above 600 °C, i.e., with 300 °C gain in thermal stability. The use of Ru as a permanent modifier allowed pyrolysis temperatures of up to 1000 °C and 900 °C to be used for Cd in the urine sample and in aqueous solution submitted to CPE, respectively. As a compromise, a pyrolysis temperature of 800 °C was adopted for further experiments; the selected atomization temperature was 1600 °C.

3.2. Complexation conditions

In order to perform a preliminary analysis of the significance of the experimental variables, a full factorial design in two levels with duplicate measurements and a central point in triplicate measurements was carried out. Four factors were evaluated, including the concentration of DDTP, HCl and Triton X-114 and the presence of the urine sample submitted to CPE, which will be from here on denominated as the "sample" parameter, for simplification purposes. The sample parameter involved the comparison between the signal obtained for an aqueous solution containing the analytes and a real urine sample enriched with the same analyte concentration. This variable was also evaluated to assure that calibration against aqueous standards submitted to CPE would be feasible. The minimum level (−) for this factor was taken as an aqueous solution containing 2.0 μg L^{-1} Pb and 0.01 μg L^{-1} Cd submitted to CPE, and the maximum level (+) consisted of a 500-μL aliquot of urine submitted to CPE, containing the same concentrations of Pb and Cd. A central point was included in the matrix of the experimental design to obtain an estimate of the variance and to check the linearity loss between the chosen levels for each variable. Table 2 contains the minimum and maximum levels for the variables studied using factorial design. The central point was taken as the arithmetic mean between minimum and maximum level values for each variable.

Treatment of the obtained data using analysis of variance (ANOVA) and statistical probability ($p=0.05$) resulted in the Pareto chart shown in Fig. 3. Fig. 3a shows that the only main effects that are statistically significant for Pb are DDTP and Triton X-114 concentrations, while Fig. 3b shows that three main effects are statistically significant for Cd. In addition, the curvature parameter, as shown in Fig. 3, was also found to be statistically significant, indicating that there is no linear relationship between the minimum and maximum levels selected for all or some of the experimental variables evaluated. This is an indication that further optimization should be carried out by means of an experimental design where at least three levels of each variable are evaluated, i.e., a response surface methodology. However, one of the most important results that can be taken from the Pareto charts in Fig. 3 is the effect of interaction between variables. For both analytes, several interactions were found to be of statistical relevance, indicating that an individual evaluation of parameters (univariate optimization) would not be appropriate as it would provide no information on interactions. Another important aspect is that the main effect of the variable "sample" was found to be not of statistical significance, which suggests that there is no significant difference at a 95% confidence level in the sensitivity obtained for the same concentration of Cd and Pb in a urine sample and an aqueous solution when both are submitted to CPE. This indicates the feasibility of carrying out calibration against aqueous standards

Table 2
Factors and levels used in the multivariate optimization

Variable	Low (−)	High (+)	Central point (0)
DDTP conc./% m/v	0.01	1.00	0.50
HCl conc./mol L^{-1}	0.01	0.63	0.32
Triton X-114 conc./% m/v	0.1 [a]/0.0 [b]	1.1 [a]/0.6 [b]	0.6 [a]/0.3 [b]
Sample volume (urine)/mL	0	0.5	0.25

[a] For Cd.
[b] For Pb.

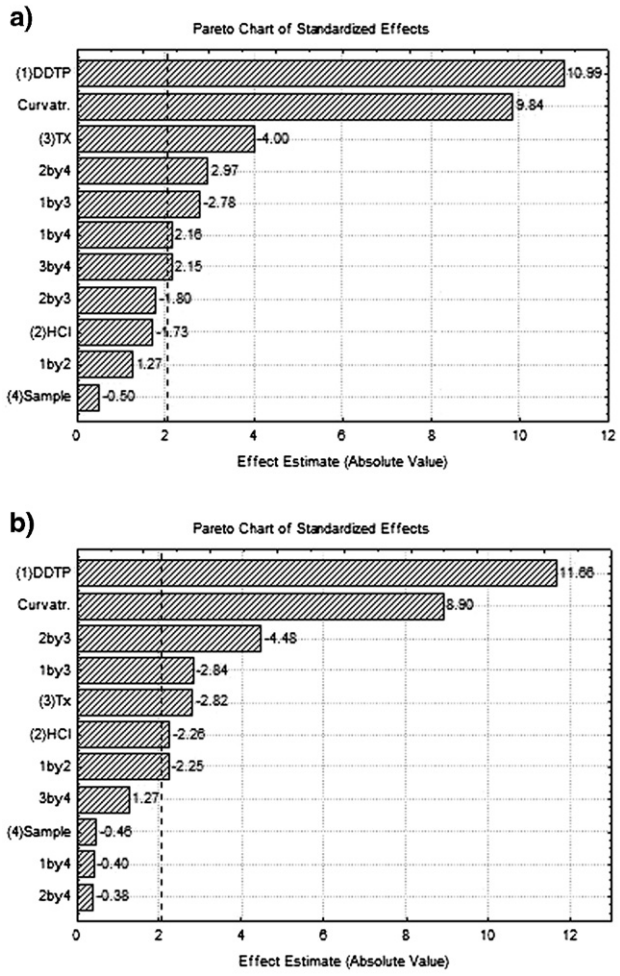

Fig. 3. Pareto charts obtained for interaction in pairs between the variables involved in cloud point extraction: (a) Pb and (b) Cd.

submitted to CPE and demonstrates that urine samples can be directly submitted to CPE without any previous acid digestion, etc., eliminating all problems associated to it, such as time consumption and potential sample contamination or analyte loss.

In spite of the fact that the presence of urine sample alone did not affect the response significantly in the procedure within the range studied, all interactions involving this variable for Pb were statistically significant, as seen in Fig. 3a, requiring a more detailed evaluation to be carried out. For Cd, no interactions with the variable sample were statistically relevant, but an optimization procedure similar to that performed for Pb was also carried out, to assure that similar responses could be obtained.

Based on the results obtained by factorial design experiments, the optimization was carried out using a response surface methodology with Box–Behnken design. Three variables were considered and simultaneously evaluated, concerning the concentrations of Triton X-114, HCl and DDTP. The optimization was carried out in parallel for Cd and Pb in urine samples submitted to CPE and in aqueous solutions, submitted to the same procedure. A total of 15 experiments were carried out in duplicate for each analyte and each matrix. Box–Behnken design allows developing a mathematical model of the analytical response as a function of the variables under evaluation. A quadratic equation is obtained, from which a critical point (maximum, minimum or saddle) can be calculated. Alternately, the critical point could also be chosen visually from the response surface.

The critical points can be determined as the points where the partial derivatives from the mathematical equations assume a null value. Therefore, from each of the equations, a total of three partial derivative equations can be taken, and each one will result in the value for maximum signal for one of the parameters involved (DDTP, HCl and Triton X114 concentrations). These values are shown in Table 3.

The equation systems obtained for Pb and Cd show that the critical points in fact correspond to maxima, therefore generating response surfaces with a maximum region visible. Fig. 4 shows the response surfaces obtained for Pb in the urine sample (Fig. 4a–c) and in aqueous solution (Fig. 4a′–c′) submitted to CPE. The behavior in both cases is quite similar, resulting in response surfaces with coincident maximum regions, confirming that similar optimum concentrations could be used for the analytes in both media. Hence, calibration against aqueous standards submitted to CPE is a feasible approach.

In all studies, the concentration of DDTP was found to be significant, as seen in Fig. 4a and a′ and Fig. 4b and b′, which is in agreement with the results of the factorial design, as shown by the Pareto chart in Fig. 3a. It can clearly be seen that there is a maximum for DDTP concentration around 0.7% m/v. The concentration of Triton X-114 is also a significant parameter, as seen in Fig. 4b and b′ and in Fig. 4c and c′. For a low concentration of Triton X-114, the signal is significantly lower probably due to poor extraction efficiency, while for elevated concentrations the analyte is more diluted in the surfactant-rich phase, and the dilution effect compensates for potentially higher extraction efficiency. It can also be seen from Fig. 4b and b′ that there is a considerable interaction between the concentrations of DDTP and Triton X-114, as both are closely related to the extraction efficiency and, therefore, to the sensitivity for the CPE procedure. The only parameter that did not show a significant influence was the concentration of HCl. Although complex formation with DDTP occurs usually in acidic media, the signal was rather independent of the HCl concentration in the studied interval. Nevertheless, to assure efficient analyte extraction avoiding formation of precipitates or adsorption on

Table 3
Conditions for maximum integrated absorbance obtained from Box–Behnken design for parameters involved in CPE

Parameter	Pb			Cd		
	Urine	Aqueous solution	Selected	Urine	Aqueous solution	Selected
DDTP/% m/v	0.70	0.77	0.70	2.46	0.93	1.00
HCl/mol L^{-1}	0.28	0.27	0.30	0.34	0.43	0.50
TX-114/% m/v	0.40	0.41	0.40	0.57	0.53	0.50

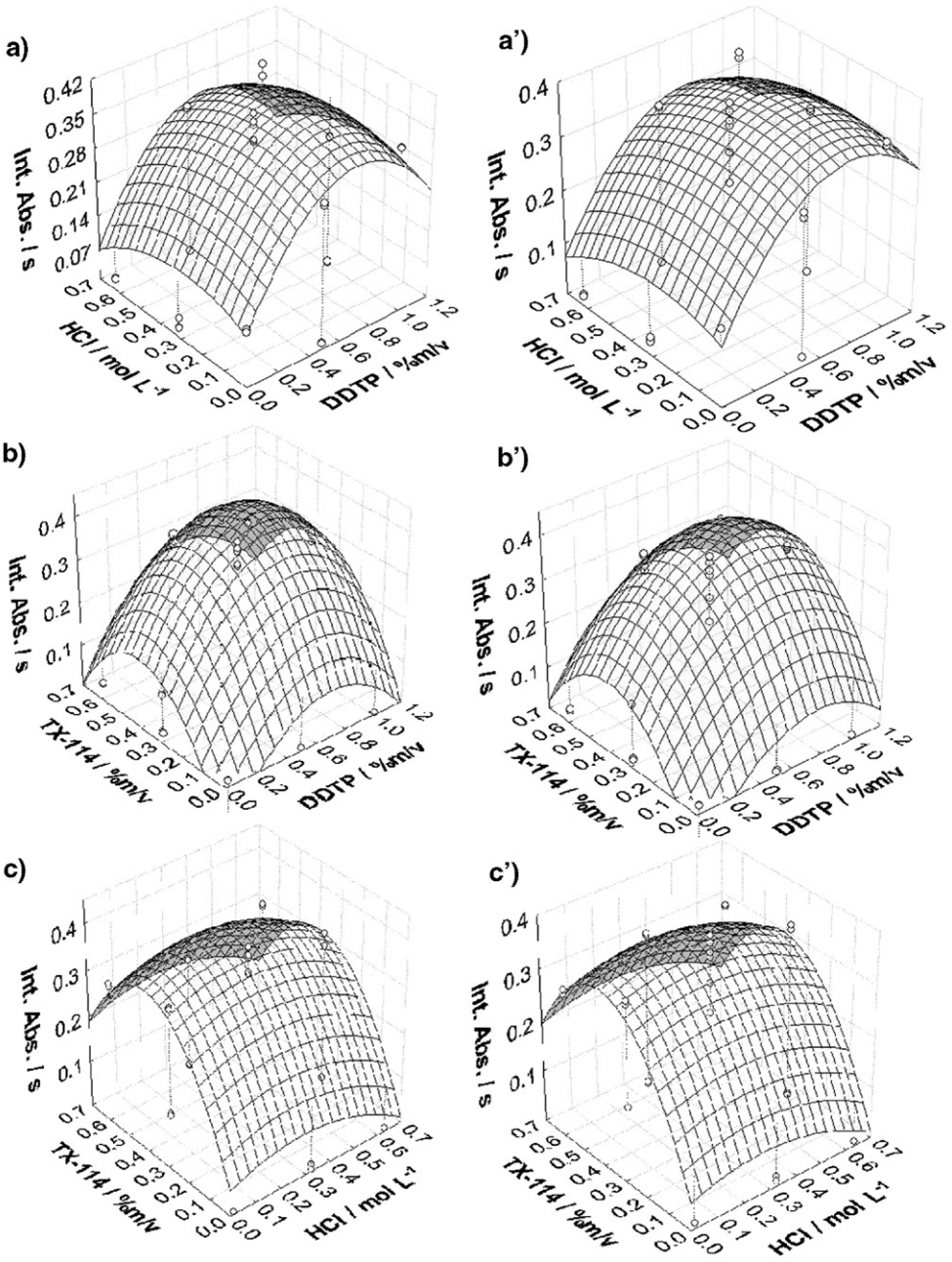

Fig. 4. Response surfaces obtained from Box–Behnken design for Pb in urine samples (a–c) and in aqueous solutions (a′–c′), after cloud point extraction.

tube walls, an HCl concentration of 0.30 mol L^{-1} was chosen, also because the signal in this region of the response surface exhibited a maximum.

The response surfaces obtained for Cd (Fig. 5) show the effect of the CPE variables for urine and aqueous solution. Although the regions of maximum signal for Cd in urine samples in Fig. 5a and b are not as clearly visible as for the corresponding surfaces obtained for aqueous standards submitted to CPE (Fig. 5a′ and b′), the maximum region is coinciding for both media, indicating that calibration against aqueous standards submitted to CPE can be used for Cd determination. The effect of increasing DDTP concentration on the analytical signal is quite pronounced for Cd in the urine sample, hence the optimum concentration value was visually chosen by interpretation of the maximum region in Fig. 5a and b. For Cd in aqueous standards submitted to CPE, the maximum region is also well defined. Unlike what has been seen for Pb, Fig. 5a, c, a′ and c′ show that HCl is in fact a statistically significant parameter for Cd in urine and aqueous solution, with increasing analytical signal as the HCl concentration increases up to about 0.4 mol L^{-1}. The signal decrease for higher HCl concentrations can be at least in part explained by the formation of charged chloride complexes, reducing the extraction efficiency. Fig. 5c and c′ also demonstrate the significant interaction between Triton X-114 and HCl, which is in agreement with the results from the factorial design, shown in Fig. 3b.

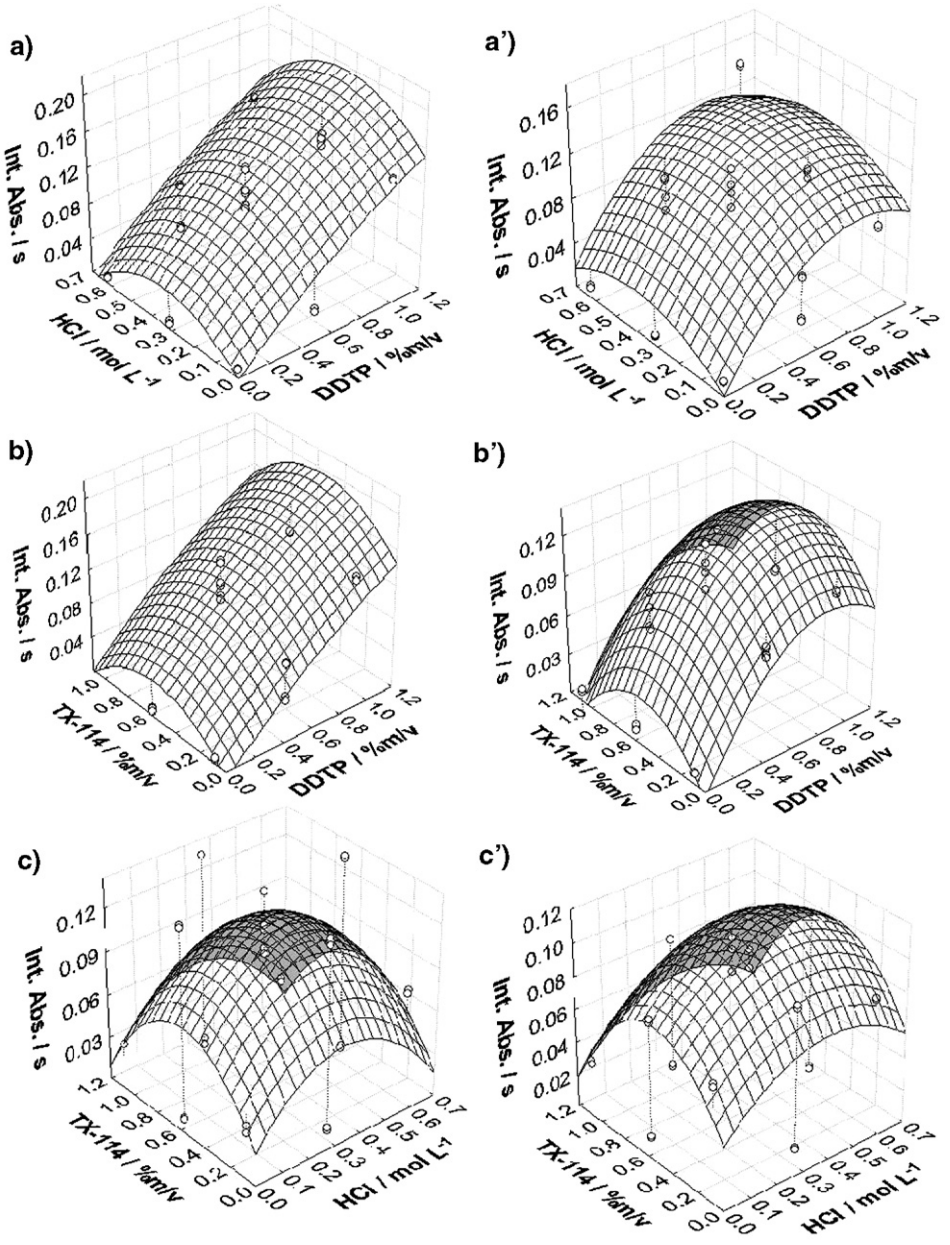

Fig. 5. Response surfaces obtained from Box–Behnken design for Cd in urine samples (a–c) and in aqueous solutions (a′–c′), after cloud point extraction.

Table 3 shows the optimum conditions obtained from the partial derivatives for Cd and Pb in the urine sample and in aqueous solution submitted to CPE. The use of these conditions results in maximum response (i.e., maximum sensitivity), according to the equations or according to visual selection in the case of the optimum DDTP concentration for Cd in the urine samples.

Table 4
Figures of merit obtained for the determination of Cd and Pb in urine samples by GF AAS following CPE with calibration against aqueous standards submitted to CPE (EF = enhancement factor; LOD = limit of detection; R = linear correlation coefficient; m_0 = characteristic mass)

	Pb	Cd
LOD/ng L^{-1}	40	2
EF	16	16
R	0.9996	0.9971
Slope/s L μg^{-1}	0.10	2.73
m_0/pg	0.5	0.03

Table 5
Concentrations (μg L^{-1}) obtained for Cd and Pb in urine control samples submitted to cloud point extraction (n = 5, Student's t for 95% confidence level)

Sample	Pb		Cd	
	Reference	Found	Reference	Found
Metalle urine level 1	130 (98–162)	147 ± 1.2	13 (10–16)	14 ± 0.3
Metalle urine level 2	80 (62–98)	68 ± 1.6	8 (6.14–9.86)	7.5 ± 0.6
Seronorm urine	91.1 ± 7	90 ± 1.4	5.06 ± 0.22	4.5 ± 0.1

Table 6
Results (μg L^{-1}) obtained for the analysis of urine samples from volunteers and spike recovery data for Cd and Pb after CPE ($n=5$; Student's t for 95% confidence level)

Sample	Pb			Cd		
	Determined[a]/μg L^{-1}	Determined[b]/μg L^{-1}	Recovery/%	Determined[a]/μg L^{-1}	Determined[b]/μg L^{-1}	Recovery/%
1	0.86±0.22	2.93±0.07	103	<0.002	0.053±0.001	106
2	0.45±0.15	2.52±0.05	104	0.051±0.019	0.102±0.002	98
3	0.14±0.09	2.30±0.07	108	<0.002	0.053±0.002	105
4	0.45±0.13	2.62±0.03	109	<0.002	0.055±0.003	110
5	0.056±0.002	1.97±0.05	96	<0.002	0.053±0.004	106

[a] Without spiking.
[b] After spiking (2.0 μg L^{-1} Pb and 0.05 μg L^{-1} Cd).

3.3. Figures of merit and results

The figures of merit are given in Table 4. The enhancement factor, calculated as the ratio between the slopes of a calibration curve for the analyte submitted to CPE and a curve without preconcentration, indicates a 16-fold improvement. Detection limits at the ng L^{-1} level were determined for both analytes, calculated from the ratio of 3 times the standard deviation of ten blank readings and the slope of the calibration curve, demonstrating the high sensitivity of the procedure. Calibration for both analytes was performed with aqueous standards submitted to the same CPE procedure as the urine samples, as justified by the multivariate optimization.

Good agreement between reference and determined values at a 95% statistical confidence level was obtained for both analytes and in all three urine control samples, as shown by the results in Table 5. Urine samples from volunteers without evident exposure to sources of Pb or Cd have also been analyzed, and the results can be found in Table 6. The natural Pb concentrations could be determined in all urine samples using CPE, while only one of the samples had a detectable Cd concentration. Spike recovery tests have also been performed using the real samples, and recoveries in the range 96-110% were obtained, demonstrating good accuracy of the method and the capability to determine Cd and Pb at trace levels in urine samples. The method has proved to be a reliable and sensitive approach to determine Cd and Pb in urine samples, particularly when compared to other procedures involving 'direct' analysis, which often require relatively complex dilution procedures and result in a reduction in the detection power [21,22]. Typically 1–3 orders of magnitude better detection limits can be obtained using CPE [17,21,22], in addition to the benefits associated to the matrix separation, reducing the risk of interference and, therefore, avoiding complex calibration procedures.

4. Conclusions

The developed procedure using CPE and GF AAS has proved to be a versatile, simple and accurate means of determining Cd and Pb at trace levels in certified and 'real' urine samples using calibration against aqueous standards submitted to CPE. The fact that no treatment of the urine samples was necessary prior to CPE certainly is a great advantage of the method, particularly concerning time saving and reduction in the risk of analyte loss and/or contamination. The use of DDTP as chelating agent and Triton X-114 allowed a satisfactory enrichment factor and low detection limits to be obtained, demonstrating the high sensitivity of the method. The use of Ru as a permanent modifier improved thermal stability for both analytes, avoiding erroneous background correction due to elevated background levels. Finally, multivariate method optimization using factorial and Box–Behnken design has proved to be an extremely valuable tool, allowing accurate optimum values of experimental parameters to be determined as well as the possibility to evaluate the interaction between variables with a reduced number of experiments. This same approach may probably be successfully extended to the determination of other analytes in urine.

Acknowledgements

The authors are thankful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), to Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and to Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) for financial support. E.M. and D.L.G.B. have scholarships and E.C. and A.J.C. have research scholarships from CNPq. T.A.M. has a scholarship from CAPES and B.W. has a research scholarship from FAPESB.

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