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Determination of lead in human saliva by combined cloud point extraction–capillary zone electrophoresis with indirect UV detection

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Abstract

A micelle-mediated phase separation without added chelating agents to preconcentrate trace levels of lead in human saliva as a prior step to its determination by capillary electrophoresis has been developed. The enrichment step is based on the cloud point extraction of lead with the non-ionic surfactant PONPE 7.5 in the absence of chelating agent. The surfactant-rich phase was diluted with acetonitrile and the resultant solution was injected directly into the CE instrument. Factors affecting the combined methodology such as surfactant-rich phase diluting agent, buffer pH and concentration, applied voltage, sample preparation and presence of additives were studied in detail. A BGE of 20 mM imidazole containing 30% acetonitrile, pH 6.20 was found to be optimal for the separation of lead from other saliva constituents. Indirect detection was performed at 205 nm. The detection limit value of lead for the preconcentration of 8 ml of saliva was 11.4 $\mu g\,l^{-1}$. The calibration graph using the preconcentration system was linear with a correlation coefficient of 0.997 at levels near the detection limits up to at least 400 $\mu g\,l^{-1}$. The reproducibility (R.S.D.) on the basis of migration time and peak area were better than 0.68 and 3.6%, respectively. The method was successfully applied to the determination of lead in human saliva.

Keywords: Cloud point extraction; Saliva biomarkers; Lead; Capillary zone electrophoresis

1. Introduction

Everyday, people are exposed to a variety of chemical contaminants in food, air and even the materials in homes and the workplace. Typically, chemical exposures are nominal, producing no recognizable health impact. But some exposures such as high lead concentrations in aging homes are a critical health concern, especially when children are exposed [1,2]. In recent years, a strong secular trend toward decreasing blood lead concentrations has been noted in the general population after many of the current environmental measures were adopted such as reduction in use of leaded gasoline [3]. As a consequence, more sensitive and reliable analytical methods for low levels lead contents are required. The most effective method for assessing chemical exposure is biomonitoring or the analysis of biological fluids like blood, urine or breath. Nevertheless, these methods generally require an invasive technique. Non-invasive saliva biomonitoring offers several promising benefits; the collection and analysis of saliva would appear to be particularly attractive for a high-risk patient population where the routine collection of blood is often made difficult. A wide range of salivary biomarkers can be measured at present. These include toxins (e.g. Pb and Cd), hormones (e.g. cortisol and DHEA), various drugs and their metabolites (e.g. cotinine), DNA, and measures of mucosal function (e.g. immunoglobulins and enzymes) [4–9].

The use of urinary Pb measurements is essentially limited to long-term occupational monitoring programs, monitoring patients during chelation-therapy and, until very recently, to clinical evaluation of potential candidates for chelation therapy. However, blood lead measurements are reflective of recent exposure and also past exposures may be represented

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Nomenclature

BGE	background electrolyte
CE	capillary electrophoresis
CPE	cloud point extraction
CZE	capillary zone electrophoresis
EOF	electroosmotic flow
PONPE 7.5	polyethyleneglycolmono-*p*-nonylphenylether

in them, as a result of Pb mobilization from bone back into blood [10].

Salivary Pb should reflect freshly absorbed exposures since saliva draws upon the circulating plasma, which is the first internal compartment into which absorbed Pb resides. In conclusion, saliva monitoring represents a novel approach with broad application for evaluating both occupational and environmental lead exposures [11–13].

The analysis of lead in biological fluids is routinely carried out using techniques such as ion chromatography, atomic absorption spectroscopy (AAS) or inductively coupled plasma mass spectrometry (ICP-MS) [14–17]. Although the ICP/MS is the most sensitive measurement of lead, during the past several years, capillary electroseparation methods have moved to the forefront of analytical chemistry as a result of their versatility, low cost per analysis and high performance and throughput [18–22]. Indeed, the instrumental availability in hospitals and clinical laboratories makes CE the technique of choice.

Capillary electrophoresis has demonstrated enormous benefits compared to traditional separation methodologies, but its major drawback is poor concentration limits of detection due to the small injection volumes (typically, <1% capillary length) and narrow optical path length. Thus, lower limits of detection are required if either immediate or long-term lead toxicity studies are being performed by CE. Consequently, a preconcentration step should be included in order to achieve accurate, reliable and sensitive results.

The use of organised amphiphilic media has attracted much attention in recent years. A scheme of preconcentration can be proposed, mediated by surfactants (CPE, cloud point extraction) instead of organic solvents [23–26]. CPE is an impressive alternative to conventional solvent extraction because it produces high extraction efficiencies and concentration factors and uses inexpensive, non-toxic reagents. The use of CPE process for extraction and/or preconcentration of metal chelates, biological and clinical samples, and environmental clean-up procedures has been reported [27–32]. From an analytical point of view, the surfactant-rich phase can be used to separate and/or preconcentrate different analytes before their injection into any hydrodynamic analytical system [23]. That is why cloud point extraction is particularly adequate for HPLC, flow injection analysis (FIA) and CE. Moreover, CPE uses surfactants that inhibit the absorption of non-polar analytes to glass surfaces. The coupling of CPE to CE [28,29] and capillary electrochromatography (CEC) [30,31] has already been reported. However, the use of CPE as a preconcentration step for CE analysis of trace metal ions without added chelating agents has not been reported before.

In view of the possibility of preconcentrating lead by CPE in the absence of chelating reagents [32], and from our previous experience, the aim of the present paper was to couple an enrichment step mediated by micelles of PONPE 7.5 to capillary zone electrophoresis with indirect UV detection for the separation and determination of lead in saliva.

2. Experimental

2.1. Reagents and solutions

One milligram per millilitre standard solution of Pb(II) was prepared from acidic dissolution of its nitrate of analytical grade purity (Hopkin & Williams Ltd., Chadwell Heath, Essex, England). Stock solutions were standardised by a chelatometric method [33].

Solution A: As it is not possible to obtain a real aqueous solution of the surfactant polyethylene-glycolmono-*p*-nonylphenylether (PONPE 7.5, Tokio Kasei Industries, Chuo-Ku, Tokyo, Japan) (cloud point below room temperature) it was experimentally convenient to prepare a stock surfactant solution as follows: 20 g of PONPE 7.5, 10 ml of 1 mol l^{-1} $NaClO_4$ (Merck, Darmstadt, Germany) and 40 ml of distilled ethanol were mixed and made up to 100 ml with doubly distilled water.

Buffer solution: A 1×10^{-2} mol l^{-1} sodium tetraborate (Mallinckrodt Chemical Works, New York, Los Angeles, St. Louis, USA) solution was prepared, obtaining the desired pH by addition of dilute $HClO_4$ (Merck) or NaOH (Mallinckrodt Chemical Works) solutions.

Background electrolyte solutions. For CZE, the BGEs were composed of 5–30 mM imidazole (Merck) (pH 5.0–6.5), obtaining the desired pH by addition of dilute $HClO_4$ with different amounts of organic modifiers.

Ultrapure water (resistivity 18.3 MΩ cm) was obtained from Barnstedt EASY pure RF water system (Iowa, USA). All other reagents and solvents were of analytical grade quality and the presence of lead was not detected within the working range. All solutions were degassed by ultrasonication (Testlab, Argentina). Running electrolytes and samples were filtered through a 0.45 μm Titan Syringe filters (Sri Inc., Eaton Town, NJ, USA).

2.2. Instrumental

A Beckman P/ACE MDQ instrument (Beckman Instruments Inc., Fullerton, CA) equipped with a diode array detector and a data handling system comprising and IBM personal computer and P/ACE System MDQ Software. Indirect UV

detection was performed at 205 nm. The fused-silica capillaries were obtained from MicroSolv Technology Corporation and had the following dimensions: 57 cm total length, 50 cm effective length, 75 μm i.d., 375 μm o.d. The temperature of the capillary and the samples was maintained at 25 °C.

The pH of the electrolyte was measured by an Orion 940 pHmeter equipped with a glass-combined electrode.

All the glass instruments used were previously washed with a 10% (v/v) HNO_3 water solution and then with ultrapure water.

2.3. Experimental procedure

2.3.1. Saliva collection

In order to minimise the possibility of contamination with food debris or cigarette and airborne particles, the subjects were asked to thoroughly rinse their mouths three times, first with 1.5% citric acid solution (a salivation stimulant) and then twice with bidistilled deionised water. Human saliva samples were collected between 8 and 10 h to reduce possible circadian contributions into Pb-free polystyrene test tube, and the specimen frozen. In order to achieve a reasonable preconcentration factor compatible with lead content in saliva, sample volume was 8 ml for all cases.

2.3.2. Preconcentration procedure

Eight millilitre of human saliva, 0.8 ml buffer borax solution 1×10^{-2} mol l^{-1} (pH 8.5) and 0.8 ml of solution A, were placed in a graduated centrifuge tube. The whole mixture was diluted to 10 ml with bidistilled water. The solution prepared was kept at 70 °C for 10 min for equilibration and then centrifuged for 5 min at 3500 rpm. After being cooled at −18 °C for 5 min the surfactant phase which had separated became a viscous gel and the aqueous phase could be poured off. Finally, a 200 μl aliquot from the surfactant rich phase were diluted up to 400 μl with acetonitrile and transferred to a CE sample vial.

Blanks were prepared with the same reagents, without the samples, undergoing an identical process.

2.3.3. Determination of lead by CZE

The electrolyte solution was prepared daily and filtered through a 0.45 μm Titan Syringe filters (Sri Inc.). At the beginning of the day, the capillary was conditioned with 0.1 mol l^{-1} NaOH for 5 min, followed by water for 5 min and then with running electrolyte for 10 min before sample injection. To achieve high reproducibility of migration times and to avoid solute adsorption, the capillary was washed between analyses with ethanol for 1 min, then sodium hydroxide for 2 min, followed by water for 2 min, finally equilibrated with the BGE for 4 min. Samples were pressure-injected at the anodic side at 0.5 psi for lengths of time 3–7 s. To avoid buffer contamination caused by adsorption of surfactant onto the outer wall of the capillary, the anodic side of the capillary was immersed in ethanol during 2 s immediately after sample injection. A constant voltage was used for all the experiments. Indirect UV detection was performed at 205 nm.

Electroosmotic flow (EOF) determination was performed by using acetone as an EOF marker. The EOF marker was prepared by diluting 1 ml of acetone with the BGE and sonication for 5 min prior injection.

3. Results and discussion

3.1. Development of the preconcentration step

In the present paper, our research aim was to expand the potentiality of the micelle-mediated preconcetration schemes by studying the feasibility to couple a CPE system without added chelating agent to capillary zone electrophoresis with indirect UV detection, able to give baseline separation of lead from other saliva's components with good resolution, reproducibility and accuracy compatible with biomonitoring analysis.

PONPE 7.5 has previously been used as extracting surfactant of metal chelates [26,27]. Nevertheless, in previous works [12,32] we have reported the CPE of unchelated metals. Quantitative lead extraction was reached without added chelating agents. This is possible because PONPE 7.5 forms a cationic complex with $[Pb(OH)]^+$ through their polyoxyethylene groups [34], while other metals present in the sample do not form stable complexes with the surfactant.

The cloud point of the studied system with PONPE 7.5 is near room temperature (18 °C), offering advantages in terms of the experimental procedure, thus avoiding a potential loss of extraction efficiency during the centrifugation step.

The effect of several experimental parameters upon the extraction parameters and sensitivity have been thoroughly evaluated and optimized.

Surfactant concentration: The effect of PONPE 7.5 concentration upon sensitivity and extraction parameters was studied within the surfactant concentration range 0.1–2.0 % (w/w). Quantitative Pb(II) extraction was observed for an amphiphile concentration higher than 0.75% (w/w). In order to achieve a good preconcentration factor, 0.8 % (w/w) was chosen as optimal.

Effect of ethanol: The presence of ethanol prior to the extraction step produces important benefits in terms of preconcentration factor ($f = v_w/v_s$, where v_w represents the volume of aqueous phase and v_s the volume of surfactant rich phase) and kinetics of phase separation. When the CPE step was performed in the absence of ethanol the volume of the surfactant rich phase, v_s was 0.30 ml and 120 min were required for complete phase separation (equilibration time). When the CPE step was carried out in the presence of ethanol (concentrations equal or higher than 0.3% (v/v), v_s was 150 μl and equilibration time 3 min. Thus, an ethanol concentration of 0.4% (v/v) was chosen as optimal.

Effect of buffer concentration and ionic strength: The optimal results were obtained with sodium tetraborate

as buffer agent. The influence of buffer concentration prior to CPE was investigated within the range: $C_{\text{sodium tetraborate}} = 0.5 \times 10^{-3}$ to 1.0×10^{-2} mol l^{-1}. Sodium tetraborate 1.5×10^{-3} mol l^{-1} was chosen as optimal.

The ionic strength has no considerable effect upon the magnitude of extraction and sensitivity within the interval $\mu = 0.0$–1.00 mol l^{-1}. Thus, the ionic strength was kept constant at 0.01 mol l^{-1} with sodium perchlorate.

Effect of pH: Experiments were made in order to locate the optimal pH range for the quantitative lead extraction. Each desired pH value was obtained by the addition of $HClO_4$ (Merck) (d) and/or NaOH (d). Lead extraction begins at pH 4.20 and starts to decrease at pH 10.00 offering a relatively wide range for quantitative extraction.

Effect of centrifugation time: A centrifuge time of 5 min was selected as optimum since complete separation occurred at this time and no appreciable improvements were observed for longer times.

Diluting agent for the surfactant-rich phase: The very high viscosity of the surfactant rich phase (20 cP approximately) was drastically decreased with a diluting agent. Different solvents for the surfactant-rich phase were tried so as to select the one producing the optimal results regarding sensitivity and CE compatibility. The best results were shown for acetonitrile. In this situation, the CPE fractions may be appropriately manipulated and injected. So, the surfactant rich phase was transferred to a CE sample vial and diluted with 100% acetonitrile up to 400 μl.

3.2. Development of the separation conditions

In order to propose a specific and accurate way of analyzing CPE-preconcentrated human saliva containing lead by using capillary zone electrophoresis with indirect UV detection, it is essential to find the best experimental conditions in which the analytes can be separated from each other. The optimization was performed using a human saliva sample spiked with a known amount of Pb.

The following parameters were consecutively optimized: sample conditioning, pH, BGE composition and concentration, sample and capillary temperatures and other electrophoretic parameters such as separation voltage, injection mode and length, etc.

Effect of pH: The buffer pH plays an important role for improving selectivity in CE especially for closely related compounds, because it affects both the overall charges of the solute and the electroosmotic flow. The effect of the BGE pH was investigated within the range 4.00–7.00 at a fixed buffer concentration, adjusted by 0.1 mol l^{-1} NaOH and 0.1 mol l^{-1} $HClO_4$. It was found that when the pH was increased, resolution also increased, while analysis time decreased. The best results were obtained for pH 6.00.

Effect of BGE composition and concentration: BGE concentration has also a significant effect on the separation performance through its influence on the EOF and the current produced in the capillary. Different BGEs have been tested, but the one producing the best results considering selectivity, reproducibility, baseline and current performance, was imidazole containing acetonitrile, pH 6.00. Keeping other parameters constant (pH 6.00, 25 kV, 25 °C) the BGE concentration was varied from 5 to 75 mM. Increases in migration times as well as current were observed when the concentration of buffer increased. Resolution also increased for higher buffer concentrations, but no appreciable improvements were observed for BGE concentrations above 20 mM.

It has been reported that organic modifiers are very important to improve separation in many systems because they can change the partition coefficient and polarity of the sample. To search for an appropriate separation medium and avoid surfactant adsorption onto the wall of the capillary, the effect of the percentage of acetonitrile in the imidazole buffer solution on the CE separation in the surfactant-rich phase was studied. Various amounts of acetonitrile (5, 10, 15, 20 and 30%) were added into the imidazole buffer, pH 6.00. Separation of lead from other saliva's components and stable baseline was obtained when 30% and of acetonitrile was added.

So, a 20 mM imidazole buffer containing 30% acetonitrile, pH 6.00 was chosen as the BGE as it gave a full separation of lead from other saliva's components in under 5 min. Fig. 1 shows the electropherogram obtained for an CPE extracted human saliva sample spiked with 40 μg l^{-1} of lead.

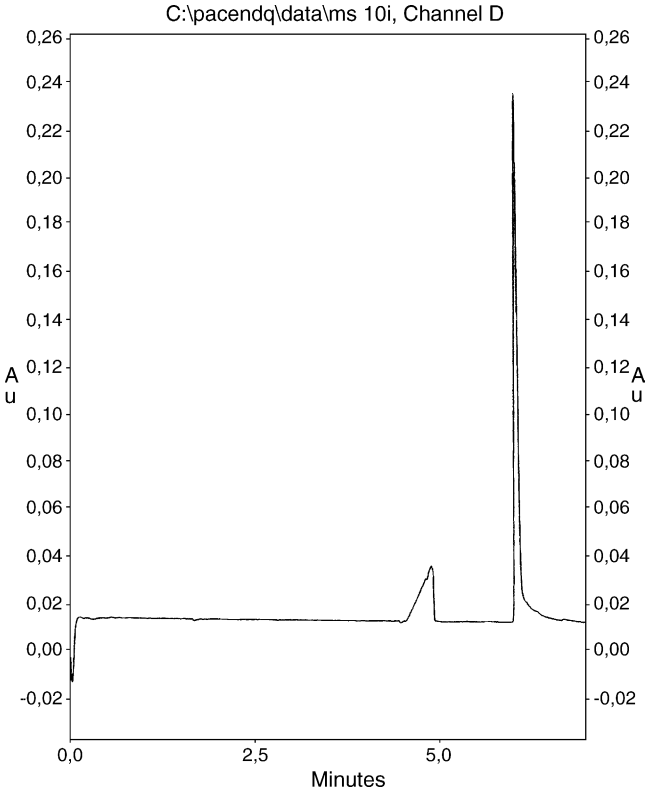

Fig. 1. Electropherogram of human saliva sample spiked with Pb(II). *Conditions*: 20 mM imidazole buffer containing 15% acetonitrile, pH 6.00; capillary, 57 cm full length, 50 cm effective length, 75 μm i.d., 375 μm o.d.; hydrodynamic injection at 0.5 psi, 5 s; 25 kV constant voltage; indirect UV detection at 205 nm.

Table 1
Recovery test

Aliquots	Base value ($\mu g\,l^{-1}$)	Pb added ($\mu g\,l^{-1}$)	Pb(II) found ($\mu g\,l^{-1}$)	Recovery (%)[a]
I			38.2[b]	–
II	38.2	14.0	52.6	102.85
III	38.2	28.0	65.9	98.93
IV	38.2	40.0	78.8	101.50
V	38.2	100.0	136.8	98.60

Conditions: Capillary, 57 cm full length, 50 cm effective length, 75 μm ID, 375 μm OD; hydrodynamic injection at 0.5 psi, 5 s; 25 kV constant voltage; indirect UV detection 205 nm.

[a] $100 \times [(\text{found} - \text{base})/\text{added}]$.

[b] Mean value, $n = 6$.

Injection: The injection mode giving the best response concerning reproducibility and linear range was the hydrodynamic mode, while the electrokinetic mode gave a slightly greater sensitivity. Injection parameters were optimized by varying the lengths of sample (3–7 s) and injection pressure until optimum conditions were reached. The best results were obtained for the following experimental parameters: hydrodynamic injection, 5 s and 5 psi.

Due to the high viscosity of the sample, buffer contamination caused by adsorption of surfactant onto the outer wall of the capillary was observed with consequent loss of separation efficiency and reproducibility. To avoid such effects, the anodic side of the capillary was immersed in ethanol during 2 s immediately after sample injection. Thus, good reproducibility was obtained.

Interference species: The effects of representative potential interfering species were tested. Cu(II), Zn(II), Cd(II), Ni(II), Co(II), Mn(II) and Fe(III) could be tolerated up to at least 2000 $mg\,l^{-1}$. Commonly encountered matrix components such as alkali and alkaline earth elements generally do not form complexes with the surfactant polyoxyethylene groups and thus are not CPE extracted.

3.3. Separation performance: evaluation of the combined methodology

An extraction percentage higher than 99.9% was achieved when the procedure was carried out under the optimal experimental conditions. Consequently, the enrichment factor achieved for this system was 25-fold.

The calibration plot was obtained representing the ratio of the corrected areas versus concentration. The calibration equation was calculated by the least-squares linear regression method, and unknown concentrations were calculated by interpolation. The detection and quantitation limits were calculated as the analyte concentrations that give rise to peak heights with signal-to-noise ratios of 3 and 10, respectively. The limit of detection of lead for the preconcentration of 8 ml of saliva, based on a signal to noise ratio of three was 11.4 $\mu g\,l^{-1}$. The calibration graph using the preconcentration system was linear with a correlation coefficient of 0.997 at levels near the detection limits up to at least 400 $\mu g\,l^{-1}$.

In order to determine the repeatability (within-day precision) of the method, replicate injections ($n = 6$) of a solution containing 50.0 $\mu g\,l^{-1}$ of lead were carried out. In all cases, the precision was better than 1.7% for the migration time and 2.4% for the peak area. Good peak area precision was achieved without adding any internal standard.

The reproducibility (between-day precision) was also evaluated over 3 days by performing six injections each day. The reproducibility (R.S.D.) on the basis of migration time and peak area were better than 0.68 and 3.6%, respectively.

Robustness of the method was performed by two analysts (six determinations) using the proposed method and the same instrumentation. The results showed no significant differences: 98.2% (found) and 1.0% (R.S.D.).

The overall time required for preconcentration of 8 ml of sample (10 m equilibration, 5 m centrifugation, 5 m cooling), injection–separation (approximately, 6 min was about 26 min; hence, the throughput was approximately 2.3 samples per hour. Nevertheless, it has to be pointed out that several samples can be CPE-processed simultaneously.

In order to validate this method, 80 ml of human saliva were collected and divided into 10 portions of 8 ml each. The proposed method was applied to six portions and the average lead concentration determined was taken as a base value. Then, known quantities of lead were added to the other aliquots, and its concentration determined following the recommended procedure (Table 1). The CPE procedure was practised in saliva samples without previous treatment.

The lead levels considered under this study were compatible with those considered by other authors, e.g. Gonzalez et al. [35] refers to lead quantities in saliva of 0.3 $\mu g\,l^{-1}$, while other studies performed in our laboratory refers to lead concentrations of 20 $\mu g\,l^{-1}$ [13].

4. Conclusions

The results for this work demonstrated for the first time the feasibility of coupling a cloud point preconcentration step without added chelating reagents to capillary zone electrophoresis with indirect detection. Substantial improvements were attained with the proposed method compared to published reports for lead determination by CE in terms of simplicity and sensitivity.

Although the limit of detection of the present approach is not compatible with saliva lead Pb content in unexposed population, it is possible to perform the recommended procedure from a larger sample volume and consequently reach lower LODs.

The recommended procedure presented herein was found to be easily applicable to the analysis of human saliva, providing a sensitive method for the determination of lead with minimal sample handling and high reproducibility. It represents a simple, rapid, low cost, non-polluting contribution in the field of sialochemistry related to non-invasive techniques of sampling.

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