Direct determination of lead in bones using slurry sampling high-resolution continuum source electrothermal atomic absorption spectrometry

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Abstract

Straightforward, quick, sensitive and reliable method is introduced for determination of lead in bones using slurry sampling and high-resolution continuum source electrothermal atomic absorption spectrometry (HR-CS-ETAAS). The spectral interference caused by the molecular absorption of PO molecules with rotational fine structure coinciding with the analyte absorption at the most sensitive resonance line 217.001 nm in time was identified and successfully corrected by applying a mathematical correction algorithm using the spectrum obtained by vaporization of hydroxyapatite. The slurry preparation and measuring conditions were determined by means of a response surface methodology. Experiments were designed according to a 2^{7-4} replicate (n = 3) fractional factorial design for seven factors (particle size, glycerol and HNO_3 concentration, sonication time, concentration of chemical modifier, pyrolysis and atomization temperature) each at three different levels, including central points. The optimized conditions were 100 mg of a ground sample with particle size < 315 μm, dilution in a liquid-phase composed by 10 % w/w glycerol, 5.0 % w/w nitric acid solutions, sonication time of 2 min and final slurry volume of 10.0 mL. Detection limit of 9.1 μg kg^{-1} and characteristic mass 7.6 pg were achieved using the suggested method under the optimized experimental conditions. Sufficient analyte stabilization was achieved by using 1 μg Pd and 50 μg citric acid. Accurate data were obtained with the use of matrix-free calibration. The accuracy of the method was established by analysing NIST SRM 1486 Bone Meal. Further, the results acquired for ten river otter samples by slurry sampling were compared with those determined after microwave-assisted digestion by inductively coupled plasma time of flight mass spectrometry (TOF-ICP-MS) to assess the accuracy of the method. The results obtained by the two methods were compared using a paired *t*-test (at 95% confidence level) and showed no significant difference. The precision of the introduced method was better than 5.5 %.

Keywords: Lead determination; Bone analysis; High-resolution continuum source AAS; Electrothermal atomic absorption spectrometry; Matrix effects; Design of experiments; Microwave-assisted digestion; Inductively coupled plasma mass spectrometry

1. Introduction

Lead determination in bone and teeth samples has a wide-ranging importance in the clinical [1, 2], environmental [3-5], forensic [6, 7] and archaeological [8, 9] sciences as these calcified tissues are especially useful for pollution assessments [3, 4, 10], nutritional and clinical status evaluations [1, 2], toxicology analysis [8] or for identification purposes [11, 12]. The bone Pb levels of individuals who were not exposed to Pb may reach [1, 4, 13-16] up to 20 mg kg^{-1}, however, the concentration is generally lower [1, 15, 16], only occasionally exceeding 10 mg kg^{-1}. Several analytical methods such as X-ray fluorescence (XRF) [13, 14, 17], proton-induced X-ray emission (PIXE) [1], inductively coupled plasma optical emission spectrometry (ICP-OES) [18, 19], two-jet plasma atomic emission spectrometry [20], inductively coupled plasma mass spectrometry (ICP-MS) alone [2, 11, 12, 21] or combined with laser ablation (LA-ICP-MS) [9, 22], hydride generation (HG-ICP-MS) [23], atomic fluorescence spectrometry (AFS) [24] or electrothermal atomic absorption spectrometry (ETAAS) [1, 3, 6, 12, 15, 16, 21, 25-32] previously assessed the validity and limitations for the purpose of quantification of Pb in both human and animal samples. Among these methods, ETAAS [1, 5, 10, 25, 27, 31-36] still represents the most commonly used one in routine analytical practice because of its high sensitivity, favourable detection limits and relatively low cost of the instrumentation. However, due to the complex matrix, laborious sample preparation, relatively low analytical concentration and high volatility of some lead species, the analysis of bone or teeth samples is difficult [1, 33, 34, 37].

Serious analytical problems related to spectral interference from phosphate matrix during ETAAS determination of Pb in bone and teeth samples at the main analytical lines: 217.001 and/or 283.306 nm, were reported by a number of authors [5, 27, 35]. To alleviate these problems attributed to PO molecular absorption, instrumentation with several different background correction systems have been employed [3, 5, 6, 10, 15, 16, 21, 25-27, 30-33, 36, 38, 39]. Among

conventional line source atomic absorption spectrometers (LS AAS), Zeeman systems using both transverse [21, 31, 33, 36] and longitudinal [3, 25, 32, 39] arrangement were found [3, 5, 16, 21, 25, 30, 33, 34, 36, 39] to be the most reliable for this purpose. However, it has been shown that under the influence of the magnetic field the rotational lines of molecular spectrum of the gaseous phosphorus monoxide (PO) have split thus making the analysis of samples with high phosphorus content when using Zeeman effect background corrections prone to errors [39, 40].

Many chemical modifiers act as efficient thermal stabilizers of phosphorus containing species thus delaying and depressing the background signal [33, 38, 41]. Lead can be then determined in bones or in phosphates matrix using LS ETAAS [25, 27, 33, 34, 38, 41] via matrix modification. In many cases, modifiers such as Pd(nitrate) [27], Pd/Mg(nitrates) [25, 34], W–Rh permanent modifier [25] had to be applied to further attenuate the interferences produced by phosphate matrix as well as to increase the thermal stability of Pb to yield reliable results for digested [25, 27] or solid [25] calcified tissues. Application of phosphate modifiers has also been widely recommended for thermal stabilization of Pb during the analysis of bone digestates [1, 3, 31], as calcium hydroxyapatite, i.e. the main component of bone matrix and an endogenous source of phosphate, was shown [1, 3, 33] to be less efficient for this purpose. Although an excessive background absorption may be associated with the use of phosphate modifiers [39, 41], these drawbacks can be controlled by using phosphate based mixed modifiers e.g. with Pd [41], Ca [21, 33] or Mg [32, 33] or by employing an adequate amount of the phosphate modifier [39].

Most of the studies presented in the literature [1-3, 5, 6, 11, 12, 16, 21, 26, 27, 29, 30, 32, 35, 36] report that the determination of metals in bone and teeth samples is usually carried out by methods involving some type of sample digestion using nitric acid and its mixtures. In several cases, except for wet digestion, preliminary time-consuming dry-ashing step is additionally required [10, 26, 35] which may however significantly affect the recovery of Pb [27, 42].

On the other hand, direct introduction of solid samples or slurries into the graphite furnace may be more efficient as it reduces the speed and price of the analysis, risk of analyte loss and contamination and does not usually involve the use of toxic and/or concentrated chemicals making it more in compliance with green chemistry trends [43-47]. In addition, it may significantly increase the detection power that is very important in this particular case as reliable analysis of Pb in tissues of non-exposed population still constitutes an analytical challenge [14, 18]. In this term, direct solid sample analysis may be an alternative to methods employing concentration/separation steps [15, 29, 37]. While these methods are usually time consuming, and prone to serious systematic errors [28], they may help to reduce the matrix effects. On the other hand, spectral interferences caused by molecular absorption due to diatomic molecules with pronounced fine structure [48] may be the major obstacle in the application of direct solid samples analysis due to the limited background correction capability especially for the LS AAS [28].

In contrast to the conventional AAS systems, HR-CS-AAS enables both detailed observations of the structured background signals as well as the efficient correction due to its unsurpassed background correction capabilities [49-52]. Previously, a number of new methods for trace elements analysis in a wide variety of complex matrices by HR-CS-ETAAS using direct solid sampling were elaborated without any interference [43, 49, 50, 53]. However, according to the best our knowledge to this date no method has been published for the purpose of direct determination of Pb in bone solids by this technique.

In this work, the development of such reliable and environmentally friendly method suited for routine direct analysis in tissues of non-exposed animals, which constitutes an analytical challenge is described. The ‘visibility’ of the spectral environment around the analytical line with the employment of the least squares background correction (LSBC) for elimination of the fine structure of PO band directly hampering the determination of Pb at the

resonance line 217.001 nm together with the response surface methodology (RSM) employed for optimization of experimental conditions has been shown to significantly facilitate the method development.

2. Experimental

2.1 Instrumentation

The analyses were performed using the model contrAA 600 high-resolution continuum source atomic absorption spectrometer (Analytik Jena AG, Jena, Germany) equipped with transversely heated graphite furnace and autosampler MPE 60. Xenon short-arc lamp with a nominal power of 300 W, operating in a hot-spot mode was the light source. The double monochromator (prism and echelle grating) combined with a CCD array detector with 588 pixels ensures the unique resolution with a spectral bandwidth of below 2 pm/pixel. Peak area absorbance values were measured. The primary analytical line 217.001 nm for Pb was used for the measurement. Pyrolytically coated graphite tubes (Analytik Jena, Part no. 407–152.023 Schunk, Germany, Batch-No: 103074348-46/13) with preinstalled PIN platform and integrated contacts for transverse heating were used. Argon (99.999 %, Linde Gas, Inc., Czech Republic) was used as the sheating gas. The internal gas flow in the graphite tube was interrupted during the atomization step and also during one part of the pyrolysis stage.

The microwave-assisted digestion of samples was performed in the Speedwave™ Xpert (Berghof, Germany) microwave system with the maximum total output of the microwave generator 2000 W. The sample throughput of the Xpert system was increased by using the Multitube System (MT) employed with the DAK-100X digestion vessels [54]. This arrangement allows simultaneous digestion of three samples in one DAK-100X PTFE vessel by placing three MT PFA tubes into each of the vessels.

Inductively coupled plasma time of flight mass spectrometer Optimass8000 (GBC

Scientific Equipment Pty. Ltd., Australia) [55] was used in several cases for comparative measurements.

Samples of slurries were sonicated using a Sonorex Super RK52 ultrasonic bath (35 kHz, RF-power 240 W; BANDELIN electronic GmbH & Co. KG, Germany).

The diffraction patterns (Cu Kα, λ = 1.5418 Å) were recorded on powdered samples using a D8 Advance diffractometer (Bruker AXS, Germany) with Bragg-Brentano Θ-Θ goniometer (radius 217.5 mm) equipped with Ni-beta filter and LynxEye detector. The scan was performed at room temperature from 5 to 70° (2 Θ) in 0.01° step with a counting time of 2 s per step.

The scanning electron microscopy (SEM) images were recorded with a VEGA3 TESCAN model under high vacuum at 20 kV accelerating voltage. Energy-dispersive X-ray spectroscopy (EDX) was used to characterize the presence of major elements in bone samples using Bruker Nano GmbH, X Flash Detector 410 model, Germany.

CoolSafe 4-15 L bench-top freeze dryer (LaboGene, Denmark) for drying of samples and enhancing both stability of a dry powder and analyte in a dry state as well was used throughout this study.

Wig-L-Bug 30 (Crescent Dental Mfg. Co.) vibration mill was used for production of powders from individual samples. Particle size distribution was measured by a Mastersizer 2000/MU (Malvern Instruments, Ltd., GB).

2.2 Reagents and standards

Lead stock solution with 1 g L^{-1} Pb in 3% HNO_3 was obtained from Analytika, Ltd. (Czech Republic). Nitric acid (65%, w/w) of Selectipur quality and glycerol (99.6%, p.a.) were purchased from (Lach-Ner, Neratovice, Czech Republic). Laboratory grade Triton X-100 (4-(1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol) and synthetic hydroxyapatite

(99.8%, trace metals basis) were obtained from Sigma-Aldrich (USA). Hydrogen peroxide (Trace Select, ≥ 30%, w/w) was purchased from Fluka (Switzerland). The solution of 1 g L^{-1} of Pd in 10% v/v HCl was bought from SCP Science (Canada). Solution of 25 g L^{-1} of citric acid (Lachema, Brno, Czech Republic) was prepared by dissolving this pro-analysis grade salt in water. Solutions were prepared using ultrapure water of 0.055 μS cm^{-1} conductivity obtained using the Milli-Q® (Millipore, USA) water purification system.

2.3 Quality assurance and quality control

Commercially supplied quality control material SRM® 1486 Bone Meal intended primarily to evaluate analytical methods used for the determination of selected major, minor, and trace elements in bone and in material of a similar matrix was purchased from National Institute of Standards & Technology, USA. Particle size smaller than 355 μm was ensured by the material certificate.

2.4 Sample collection, storing, handling and preparation

Ten rib samples from Eurasian otters, mostly killed in traffic accidents, were obtained from the ALKA Wildlife organization, which is a group of experts on ecology and conservation of wildlife in the Czech Republic. In addition for method development purposes, rabbit bones were obtained from a local farm.

At first, all adhering tissues and tendons were removed from the samples (Fig. S1a,b) with a ceramic knife made from zirconium dioxide. After that, bones were cut into smaller pieces (Fig. S1c,d) and marrow deposits were removed by a stainless steel scraper.

Around 1 g of the sample was placed into the containers (Fig. S1c) wherein the material was dried. The containers were tightly closed, and the material was subsequently freeze dried in the closed container under the conditions developed and tested successfully

with animal bone tissues in order to allow for sample variability. Before the freeze drying process, the samples were placed in a deep freezer at −80 °C for 24 hours to provide a necessary conditioning for low temperature drying. Total drying times of about 29 hours were employed, with the room temperature held in the first drying stage for about 1 hour and a total chamber pressure of 0 Pa, followed by a 24-hour drying stage at –111°C and 0 Pa and a final drying stage at room temperature and 0 Pa pressure for 4 hours.

The dried samples were placed in a stainless steel vial (1" height x 1/2" diameter) with stainless steel ball pestle (1/4" diameter) (Fig. S1d) and ground in a vibration mill. The powder (Fig. S1d) was then sieved manually by using sieves with hole sizes 315, 160 and 54 μm. Ground bone samples were kept in sealed plastic vials and stored at –20 °C until analysis.

2.5 Procedure for slurry analysis

The slurries were prepared using the following procedure. An accurately weighed amount of about 100.0 mg of sample (fractions < 315, 160 or 54 μm) was transferred into a 10 mL calibrated flask and three drops of 2% (w/w) Triton X-100 solution in ethanol were added to ensure wetting of the sample. Appropriate amounts of glycerol and/or nitric acid were added to yield a final solution containing 0, 10, 20 % (w/w) glycerol and 0, 2.5 and 5 % (w/w) HNO_3. The slurries were then sonicated for 2, 6 or 10 min in an ultrasonic bath. Thereafter, approximately 1 mL of the slurry was transferred into the autosampler cup and then only manual shaking was performed a right before the injection. Finally, 25 μL of the slurry plus 0–10 μL of the chemical modifier mixture containing 0.5 g L^{-1} of Pd and 25 g L^{-1} of citric acid were injected into the graphite furnace by the auto-sampler and subjected to the heating program presented in Table 1. The preparation of the reagent blank was subjected to the procedure as outlined above, to correct for any possible trace amount of the analyte in the reagents used for slurry preparation. The slurries, similarly as all the other samples

investigated in this study, were prepared in triplicate.

The concentrations of five standard solutions used to obtain the calibration curves ranged from 0 to 40 μg L^{-1} of Pb. The calibrations and standard additions were controlled by the instrument software. Next to the aqueous calibration, two standard additions were made.

2.6 Procedure for microwave digestion

For comparative purposes, an adapted microwave-assisted procedure [56] was used. Sample mass of 100.0 mg was weighed into the MT-tubes and 1 mL of 65 % (w/w) HNO_3 and 1 mL of 30% H_2O_2 (w/w) were added. These MT-tubes were placed into the outer digestion vessel containing 15 mL of HNO_3 (65%, w/w) and H_2O_2 (30%, w/w) mixture (1:1, v/v). This ensured that the level of the HNO_3-H_2O_2 mixture was higher in the outer vessel than in the PFA tubes. The vapour pressures were thus compensated and the evaporation of the solution from the PFA tubes was forestalled [54]. Samples were digested according to following 5-steps program: (i) 10 min at 130 °C and 20% power (ramp 5 min), (ii) 10 min at 160 °C and 40% power (ramp 5 min), (iii) 15 min at 200 °C and 60% power (ramp 5 min) (iv–v) 5 min at 50 °C and 0% power (ramp 1 min). The resulting solutions were diluted to 10 mL with deionised water.

Determination of Pb in the mineralized samples by a reference TOF-ICP-MS method was done using aqueous standard calibration curve with Rh as an internal standard [56].

2.7 Experimental design and statistical data treatment

The response surface methodology [57-59] was used to estimate the main effects of the selected variables onto analytical response and to find the optimal conditions for the sample preparation and measuring conditions as well. The variables selected in this study were: (1) glycerol concentration (%, w/w), (2) HNO_3 concentration (%, w/w), (3) time of sonification,

(4) size of particles, (5) pyrolysis temperature, (6) atomization temperature, (7) concentration of chemical modifier.

The Box, Hunter, and Hunter [57, 58] two-level (2^{7-4} + 3C, n = 3) fractional factorial design (FFD) [57, 58] was used for designing the experimental data. Thus, only 33 experiments were run instead of 384 required for a full factorial design (2^7, n = 3) as the number of experiments in FFD is given by ($2^{k-p} \times n + C \times n$), where k is the number of variables, C is the number of central points, n number of replicates and p a whole number that indicates how fractionated the experimental design is. All factors were evaluated at two levels, low (denoted as −1) and high (denoted as +1). The central point of the design space (middle value denoted as 0), i.e. the experiment, in which all the parameters have a value which is the average between the low and high level, was also added to the experiment. The investigated maximum and minimum levels of the variables are shown in Table 2. The responses for each experiment calculated as the mean of integrated absorbance obtained for Pb in SRM® 1486 Bone Meal are summarized in Table S1.

The experimental data were processed using the Statistica 12 computer program (StatSoft, Inc., USA), Minitab 18.1 (Minitab Inc., USA) and QC Expert™ 2.5, TriloByte Statistical Software, (Pardubice, Czech Republic).

3. Results and discussion

3.1 Evaluation of spectral interference on Pb determination

Spectral interference may be recognized and controlled by using the visibility of the spectral environment at high resolution in HR-CS-AAS as a diagnostic tool [48]. The wavelength-resolved absorbance spectrum as collected on contrAA®600 during the atomization of a slurry of NIST SRM 1486 Bone Meal shown in Fig. 1a demonstrates the well-structured background in the surroundings of the most sensitive analytical line of Pb at

217.001 nm. This complex background may be attributed to the diatomic molecule PO caused by vaporization of bone matrix as this in agreement with the reference spectrum recorded with hydroxyapatite (Fig. 1b). The structured background caused by PO molecules is thus recognized to be the main spectral interference in the determination of Pb in bone samples by ETAAS. No other potential interferences in the neighborhood of the lead line at 217.001 nm by e.g. a secondary aluminium line at 216.883 nm and a secondary iron line at 217.130 nm were observed, although these have been reported [60] previously for determination of Pb in various biological samples.

The presence of structured background absorption caused by PO makes the determination extremely difficult or virtually impossible using LS ETAAS, as none of the currently available background correction systems of such instruments is able to correct reliably for this kind of absorption [40]. However, as it is evident from the literature [40, 48, 60], this interference can be fully controlled by HR-CS-ETAAS during the determination of several analytes in various matrices under optimized conditions. It is important to highlight, that chemical composition of each sample is responsible for the fine structured background [33, 38-40] and even for the same analyte in a similar matrix the situation may be quite different.

The HR-CS-ETAAS instrument software enables to correct the fine-structured background caused by the interfering molecules by means of the least-squares background correction (LSBC) using reference spectra [48, 50]. However, when the PO bands do not overlap with the analytical line it is not necessary to employ LSBC because of the high spectral and time resolution of the equipment the atomic absorbance signal can be resolved in time from the molecular structures by the setting integration limits adjusted to integrate only the atomic signal [48, 60]. While, using this approach the interference-free determination can be achieved for Pb at 217.001 nm line using direct solid sample analysis in a variety of biological materials [60], the situation for determination of Pb in bones is more complex. In this case a direct

289 coincidence in time between the atomic and molecular absorption is observed (Fig. S2) using
290 the atomization temperature of 1500 °C and higher. Although, according to the literature [48],
291 the phosphates in biological materials, which are the source of interfering PO molecules, are
292 volatilized only above 1700 °C it can be seen from Fig.S2 that background absorption with
293 pronounced fine structure is observed even at lower temperatures. These observations are also
294 in accordance to those published previously by Borges at al. [60]. Lowering the atomization
295 temperature to less than 1500°C may lead to problems associated with broad transient signal
296 resulting in worse precision and longer atomization step.
297 Reference spectrum belonging to the molecule causing the spectral interference is
298 necessary for successful elimination of the fine structured background by its subtraction using
299 LSBC [40]. In the literature, PO spectra obtained by vaporization of $NH_4H_2PO_4$ [40] or
300 $(NH_4)_2HPO_4$ are most widely employed as a reference. However, thermal behaviour of these
301 compounds [61] is significantly different compared to bone phosphate and thus for generation of
302 reference spectra hydroxyapatite was used.
303 The PO reference spectra were recorded using 20–250 μg of hydroxyapatite which
304 was introduced into the graphite furnace via the injection of 2–25 μL of 1.0 % (w/w)
305 $Ca_5(OH)(PO_4)_3$ slurry together with the chemical modifier. The reference spectra were stored
306 in the method for subsequent subtraction from the sample spectra. During the analysis of real
307 samples the reference spectra recorded using 200 μg of hydroxyapatite removed essentially all
308 the molecular absorption structures, as is shown in Fig. 1b. The robustness of the method to
309 correct for structured background is documented (Fig. S3) for up to 250 μg of $Ca_5(OH)(PO_4)_3$,
310 which corresponded to the hypothetical situation that the slurry samples of 1 % (w/w)
311 concentration prepared according to the description in section 2.5 would contain nothing but
312 hydroxyapatite. The robustness of the method for the correction of spectral effect is important
313 as the composition of bone matrix may vary among the analysed samples as can be seen from

the results of XRD (Fig. S4) or EDX (Table S2) analysis. As can be seen from the data presented in Table S2, the bone matrix is rich not only in phosphorus but also in Ca, Mg or Na. As the presence of Ca and Mg significantly affects the thermal behavior of interfering molecules and also delays the appearance of Pb [33, 39, 40], a strong and fine-structured background coinciding in time and wavelength with the analytical line of Pb 217.001 nm may be attributed just to a coexistence of a high concentration of P and Ca, eventually also with Mg.

3.2 Optimization of the experimental conditions for slurry analysis

Variables including particle size, pyrolysis and atomization temperature, volume of chemical modifier, concentration of stabilizing and extracting agent, and ultrasonic agitation and their influence onto the analytical results was investigated using the response surface methodology for data designed by Box Hunter & Hunter [57, 58]. Each independent variable, being previously reported [44, 62] as the most important in terms of influencing the accuracy of the analytical procedure when using the slurry technique, was tested at a high (+) and a low (−) level, as shown in Table 2. The ranges of values are in agreement with practice in slurry sampling analysis and our preliminary experiments [52].

The Pareto chart depicted in Fig. 2, where the vertical line that corresponds to the 95% limit indicating statistical significance, visualizes the effects and significance of the variables. This figure reveals that the glycerol and nitric acid concentration, pyrolysis temperature and chemical modifier appeared to have a significant effect, while the other variables (extraction time, particle size and atomization temperature) were not significant factors in the studied range.

Response surface regression analysis employed to describe the data presented in Table S1 revealed that a second- order polynomial model as shown in the following equation

was the best to fit the data: $y = 0.1853\ (1.17\times10^{-2}) + 0.01417\ (1.04\times10^{-3})\ x_1 + 0.011953\ (9.3\times10^{-4})\ x_2 - 0.000123(9\times10^{-6})\ x_3 - 0.004575\ (5.80\times10^{-4})\ x_4 - 0.000733\ (3.1\times10^{-5})\ x_1^2$, where *y* stands for the predicted integrated absorbance, x_1 through x_4 stand for the settings of the glycerol and nitric acid concentration (x_1, resp. x_2), pyrolysis temperature (x_3) and volume of the chemical modifier (x_4). Standard deviations of the estimates are given in parentheses. The equation illustrates the reduced quadratic model obtained from the analysis by eliminating the terms found statistically insignificant. The elimination of statistically insignificant terms helped to increase the capability for precise predictions from the model. The results of analysis of variance which is essential to test the significance and adequacy of the model are presented in Table S3. Significance was evaluated by determining the probability level that the *F*-statistic calculated from the data is less than 5%. Data given in this table demonstrates that the model is significant at the 5% confidence level since *p* values are smaller than 0.05. The large *p* value for lack of fit (>0.05) presented in Table S3 shows that the model is valid. The model adequacies were checked by R^2, adjusted-R^2 and predicted-R^2. The coefficient of determination (R^2) of the model was 0.983, which indicated a good fit between predicted values and the experimental data points. In addition, this implies that 98.3 % of the variations for analytical response are explained by the independent variables, and this also means that the model does not explain only about 1.7 % of variation. Both R^2 and predicted R^2 values obtained (see Table S3) proved the goodness of fit of the regression model.

As is depicted on Fig. 2, the analytical response is significantly improved by increasing the HNO_3 concentration and, at the same time by decreasing the concentration of glycerol and volume of chemical modifier or pyrolysis temperature. While an increase in the glycerol concentration up to 10% has a promoting effect onto the analytical response, the effect of a higher investigated concentration was the opposite. When using 10% glycerol

concentration, the suspension can be stabilized for more than 10 s, which is long enough to ensure a representative aliquot of the sample to be introduced into the cuvette after its manual shaking right before the injection. The presence of nitric acid in the slurry solution within all the investigated range affected significantly the rate of the analyte extraction and improved the precision of the measurement as well. On the other hand, the further effect of sonication treatment at different time intervals has not been shown to have a significant promoting effect onto sensitivity. Sufficient analyte extraction and slurry homogenization was thus achieved even at the lowest sonication times.

In the analysis of bones, the particle size in the range from approx. < 54 to 315 μm did not affect the analytical response (see Fig. 2), although in general the benefit from smaller particles sizes, especially for refractory and more dense samples is frequently mentioned in the literature [44] as it can positively influence the accuracy of the measurement. For otter and rabbit samples only 3 min of grinding resulted in a fine and totally ground powder ensuring that 90% of total volume was composed by particles lower than 175 μm (see Fig. S5,6). The particle diameter medians of both samples were about 50 μm (Fig. S5).

Palladium with citric acid chemical modifier, currently being well established in our laboratory [63-65], was applied to promote the thermal stabilization of the analyte. As it can be seen from Figure S7, already 1 μg of Pd with 50 μg of citric stabilizes Pb up to 1300° C, similarly as some of other chemical modifiers, which were previously suggested for this purpose [41, 66]. Although the presence of a chemical modifier during the real sample analysis impacts positively the sensitivity and the precision (see Table S1), higher amounts of chemical modifier leads to gradual decrease of the analytical response meaning that the lowest investigated amounts are adequate for the analyte stabilization. This behavior is probably the consequence of the over-stabilization of Pb by the modifier [67].

3D surface plots for overall response desirability were built to show the effects of two

variables within their studied ranges and to visualize the tendency of important factors to influence the analytical response (see Fig. 3). The desirability values for the analytical response (integrated absorbance) were set 0 for minimum (0.0017), 0.5 for middle (0.07465) and 1.0 for maximum (0.1476). These values were set based on the experimental data presented in Table S1. The individual desirability score of 1.0 selected as the target value for the optimization of the dependent factors shows its optimum value. The optimum conditions for the most important factors were as follows: 10 and 5 % (m/m) of glycerol and HNO_3, respectively, pyrolysis temperature 1100 °C and 2 μL of chemical modifier mixture containing 0.5 g L^{-1} of Pd and 25 g L^{-1} of citric acid. The optimal values for atomization temperature, sonication time, particle size and/or grinding time were set at 1600 °C, 2 min, < 315 μm and 3 min, respectively.

3.3 Figures of merit

The slope of the standard additions curve for Pb concentration was found to be quite comparable with the slope obtained by the external calibration technique over a range where the response is linear since under the optimized conditions they differ less than 10 %. These results demonstrate that the quantification of Pb in investigated samples by slurry sampling can be performed using the external calibration technique with aqueous standard solutions. This yields a model described by the following equation: $Q_A = 1.20\times10^{-2}$ ($2.64\ 10^{-4}$) [Pb] – 6.43×10^{-5} ($8.39\ 10^{-6}$) $[Pb]^2$ (where Q_A = integrated absorbance and [Pb] is the concentration of the analyte in μg L^{-1}; standard deviation of the estimate is given in parentheses). R-squared which indicates the percentage of variation that can be explained by the regression equation equals 99.99 %.

The limit of detection (LOD) and limit of quantification (LOQ) expressed as the concentration given by an integrated absorbance corresponding to three times and ten times of

414 the standard deviation of ten measurements of a sample blank were 0.091 μg L^{-1} and
415 0.30 μg L^{-1}, respectively. These values are equivalent to 9.1 μg kg^{-1} and 30 μg kg^{-1} of Pb in
416 the original sample and are sufficiently low for quantification of lead concentrations in human
417 and animal bones even for a non-exposed population [4, 68, 69].
418 The characteristic mass, which gives an integrated absorbance of 0.0044 s, was found to be
419 7.6 pg. This value is coherent with those of 5.6–8 pg presented elsewhere [40, 48, 60] for Pb at the
420 most sensitive analytical line of 217.001 nm using HR-CS-ETAAS with transversely heated
421 graphite tube atomizer.
422 The figures of merit obtained in this work at 217.001 nm (m_0 = 7.6 pg, LOD 9.1 μg kg^{-1}) are
423 better than those reported in the literature for Pb determination in bone and teeth samples by
424 LS ETAAS at 283.3 nm. The values range between 12.9–57.2 pg and 22–600 μg kg^{-1} for
425 characteristic mass value and limit of detection, respectively, using direct analysis of the
426 samples [25] or acid digestion as the sample treatment [1, 26, 27, 31-33, 39].
427 The line 283.3 nm, although less sensitive, is usually preferred [1, 27] in routine analytical
428 practice because it is less interfered by PO molecular structures [48, 66]. The proposed method
429 achieved nearly the same LOD value as those of 10 μg kg^{-1} reported by Borges et al. [60] for
430 determination of Pb at 217.001 nm in various biological samples like human hair, bovine
431 muscle, dogfish liver, pig kidney, lobster hepatopancreas, oyster tissue and bovine blood by
432 high-HR-CS-ETAAS with direct solid sampling.
433

434 **3.4 Accuracy and precision**

435 The reference material NIST® SRM® 1486 Bone Meal, with a certified content of
436 lead being 1.335 ± 0.014 mg kg^{-1}, was used to study the accuracy of the introduced method.
437 Three replicates of this material were analyzed and the mean value with 95% confidence level
438 obtained was 1.34 ± 0.08 mg kg^{-1}. As confirmed by a *t*-test (95% confidence level), no

significant difference was found between the certified and the determined values (*t*-test, 95% confidence level).

The method accuracy was also checked by analyzing real samples and comparison of the data obtained by the proposed method with those acquired after microwave assisted digestion followed by TOF-ICP-MS analysis (see Table 3).The concentration of Pb in the real samples varied from 0.28 to 2.05 mg kg^{-1}. The *p*-value for the paired *t*-test being 0.702 suggests that at the significance level of 0.05 the compared results obtained by both methods showed no statistical difference.

A linear regression analysis of the results obtained with TOF-ICP-MS and HR-CS-ETAAS using the method of weighted least squares [70] yielded a slope of 0.914 (95% confidence interval CI 0.76–1.07), intercept of 0.061 (95% CI -0.018–0.140) and $R^2 = 0.990$. These results demonstrate that the calculated slope and intercept do not differ significantly from the values of 1 and 0, respectively, and that the results achieved by both procedures are comparable.

The precision of the method was assessed in terms of intra-day and inter-day comparison. The analysis of the slurry samples three times during the same day was done to assess the intra-day precision. Inter-day precision was calculated after the analysis of the same samples on three different days during one week. Within each series, every sample was analysed in three replicates to assess the relative standard deviation (RSD). The precision of the method was found satisfactory as the RSD values of intra-day and inter-day studies were typically found to be below 5.5 %, which can be seen in Table 3.

4. Conclusions

It was demonstrated in this work that under optimized experimental conditions, an interference-free, accurate and precise determination of Pb in bone samples can be

successfully performed using HR-CS-ETAAS, slurry sampling analysis, Pd with citric acid chemical modifier and calibration using aqueous standard solutions. The fine-structured background observed at 217.001 nm caused by the presence of PO molecules, was completely corrected by employing LSBC. The spectrum obtained by vaporization of hydroxyapatite was required to reproduce the matrix and generate the correct reference spectrum. The attained detection limit of 9.1 $\mu g\ kg^{-1}$ was low enough to perform reliable Pb determinations in tissues of non-exposed animals. The results gained using the described method are comparable to those achieved using a method employing microwave assisted acid digestion. Another important advantage of this method is the use of diluted nitric acid to extract the analyte from the bone samples, which reduces safety problems and analysis costs. It may be expected that the procedure will furthermore allow simple reliable analysis of some other trace elements in calcified tissues strongly sensitive to the presence of phosphate matrix without any extensive sample preparation. In comparison with other reported methods in the references which were used for direct analysis of solid samples to determine Pb in calcified tissue samples, such as XRF, PIXE or LA-ICP-MS, the presented method offers a better limit of detection and good analytical precision for low concentration levels of the analyte, does not need to prepare pellets, has a small dependence on the size and structure of the particles to be analyzed, is interference-free, inexpensive and does not need an internal standard. On the other hand, it lacks when compared in terms of linear dynamic range or capabilities for performing depth profiling analysis, elemental mapping or in vivo measurements.

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490

Figure captions

Fig. 1. Wavelength-resolved absorbance spectra in the vicinity of the 217.001 nm analytical line using HR-CS-ETAAS recorded for NIST SRM® 1486 Bone Meal (a) without correction and (b) after correction using LSBC and PO as a reference spectrum in the presence of 1 μg Pd + 50 μg citric acid. Pyrolysis and atomization temperatures were 1000 and 2400 °C, respectively.

Fig. 2 Pareto chart of the standardized effects in the fractional factorial design 2^{7-4} for the study of variables (1) glycerol concentration, (2) HNO_3 concentration, (3) sonification time, (4) particle size, (5) pyrolysis temperature (Tp), (6) atomization temperature (Ta) and (7) volume of chemical modifier. The L and Q letters indicate linear and quadratic effect of the factor, respectively.

Fig. 3. Response surfaces from 2^{7-4} design for the desirability produced the best absorbance (target is maximized) of Pb in the bone slurry as a function of glycerol and nitric acid concentration, pyrolysis temperature (Tp) and volume of the chemical modifier.

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