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Cryogenic sample grinding for copper, lead and manganese determination in human teeth by slurry sampling GFAAS

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A simple method is proposed for copper, lead and manganese determination in deciduous teeth by graphite furnace atomic absorption spectrometry (GFAAS) using slurry sampling introduction and cryogenic sample preparation. Teeth samples were ground in a cryogenic mill in two steps: pre-cooling (5 min) and cryogenic grinding (2 min) in liquid nitrogen. After grinding, 90% of the sample particles were lower than 150 μm. The minimum mass necessary for slurry preparation as an indicator of sub-sample homogeneity was evaluated by weighting masses between 5 and 20 mg directly in autosampler cups, followed by addition of 1 mL of a solution containing 0.04% Triton® X-100 and 0.2% v/v HNO_3. Samples (20 μL) were sonicated during 20 s, before delivering into a W–Rh coated platform. Detection limits based on integrated absorbance were 34.0 ng g^{-1} Pb, 7.4 ng g^{-1} Mn and 18.0 ng g^{-1} Cu for 2% m/v slurries. W–Rh permanent modifier permitted calibration against aqueous standards. The Certified Reference Material (H-5 animal bone) from the International Agency of Atomic Energy (IAEA) was analyzed to determine lead for method validation. For copper, lead and manganese, 12 human teeth samples were analyzed using the proposed method with calibration against aqueous solution and using a comparative Pd/Mg method with the same digested samples, and standard addition calibration, with no statistical difference at 95% level on applying the *t*-test.

Introduction

Recent advances in spectrometric techniques have enabled the assessment of trace elements and their role in human health. Clinical sample analysis has been used to diagnose metal deficiency and toxicity related to human nutrition and environment exposition.[1] Lead is a non-essential metal for humans that can produce deficits in psychological functions, including intelligence, learning ability, perception, hyperactivity, and as has been suggested, even as a factor in criminal behavior.[2,3] On the other hand, copper and manganese are essential metals for a variety of biological and physiological functions in humans. They are a component of many important enzymes.[4,5] The lack of copper is associated with Wilson's and Menke's diseases, with an effect on important neurochemical functions and changes in several metabolic risk factors associated with heart disease, such as elevated plasma triglycerides, cholesterol, and uric acid.[6] Therefore, it is very important to assess these metals in human populations to evaluate both the level of body supply, with essential elements, and the degree of body poisoning, with toxic elements.

Usually, the assessment relies on blood, hair or urine assays, depending on the metal under study and its metabolic pathway. After intake, metals concentrate in many human tissues, but mainly in hard tissues, such as bone and teeth. For instance, 94% of the body burden for lead resides in bone.[7]

Due to non-invasive procedures for sample collection, deciduous tooth analysis for metal determination seems to be a very attractive way for monitoring children's metal exposition and nutrition and could be used together with other clinical samples for health information. Beyond, there is considerable evidence to show that elements are incorporated into the dental tissues forming at the time of their exposure. Therefore, unlike with bone, in which the mineral phase is subject to turnover, teeth, once formed, provide a permanent cumulative and relatively stable record of environmental exposure.[8] According to Brown *et al.*,[9] dental analysis could be used for monitoring nutritional status and environmental pollution, to investigate both the geographical variations of trace element exposure and the influence of certain trace elements on dental caries. Both permanent and deciduous teeth have been analyzed and some previous studies compared the trace element contents of these two samples.[10–14]

Owing to its high sensitivity, selectivity and ease of operation, graphite furnace atomic absorption spectrometry (GFAAS) has been frequently used for determination of many elements in clinical samples. However, tooth analysis requires a sample preparation step before the introduction into the graphite atomizer. Usually, a sample digestion step is required for tooth analysis by GFAAS,[15] but it is time consuming for a large routine scale analysis. Alternatively, solid sample analysis can be used with specific equipment in solid sampling graphite furnace atomic absorption spectrometry (SS-GFAAS),[16] in electrothermal vaporization inductively coupled plasma mass spectrometry (ETV-ICP-MS)[17] or in laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS)[18,19] without previous sample decomposition. Those techniques are also attractive for clinical applications for monitoring purposes, but most of them are cost-prohibitive or not yet of widespread use in many laboratories in developing countries.

The slurry sampling technique has been extensively used for solid sample introduction into the graphite atomizer in order to overcome the sample decomposition step. With this technique, the powdered sample is suspended in a solution containing diluted acid and a suitable surfactant before introduction into the atomizer. As the sample digestion step is not needed, sample manipulation is minimized, avoiding sample contamination. Recently, the main variables, advantages and drawbacks of the slurry sampling technique for GFAAS were reviewed.[20]

The choice of grinding technique can vary, depending on the properties of the sample matrix, especially on its hardness, fiber

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and fat contents. The most common grinding techniques employed for sample preparation in slurry sampling GFAAS analysis often result in particles with diameter less than 100 μm,[21,22] but particles as large as 500 μm can be acceptable.[23,24] Nevertheless, the time required for these procedures can be long, mainly for hard tissues. In conventional grinding techniques, such as mortar and pestle and ball mill, the hardness of tooth samples generally restricts the speed of the process for particle size reduction, generating inhomogeneous particle size distribution with particles frequently larger than 500 μm.

In slurry sampling it has been demonstrated that working with smaller particle sizes and narrowed particle size distribution could minimize errors.[20,25] Furthermore, very small sample volumes are injected into the atomizer. For instance, in 20 μL of a typical slurry containing 10 mg mL^{-1}, only 200 μg of sample are delivered into the atomizer. Then, to avoid sample inhomogeneity problems, it is necessary that the solid sample be ground in a way that ensures homogeneous particle size distribution for a representative sample mass injection into the graphite furnace.

In 1977, Iyengar and Kasperek[26] introduced a cryogenic grinding technique (brittle fracture technique) for biological sample preparation. The cryogenic grinding relies on an increase of hardness of all tissues, the insertion of failures in the (crystal) structure, and use of very smooth brittle force for the reduction to small pieces.[27] Since this time, a large number of applications of cryogenic grinding have been described.[28] However, there are few investigations evaluating the use of the technique in sample preparation employing wet digestion[29,30] or slurry preparation procedures[22,28,31,32] for elemental analysis in atomic spectrometry.

In GFAAS, in spite of the higher thermal stabilization of the analyte during the pyrolysis step due to analyte occlusion in the solid matrix, when slurry sample introduction is adopted the use of a matrix modifier is generally mandatory in order to achieve better recoveries and signal analyte stability.[33] In 1998, a W–Rh coating was proposed as a permanent chemical modifier in THGA™ for Se, Pb and Cd determinations in water by graphite furnace atomic absorption spectrometry, showing remarkable advantages when compared with traditional conventional modifiers.[34] This permanent modifier was then successfully applied for arsenic, cadmium, lead and copper determination in sediment and biological slurries,[35–40] increasing the tube lifetime and reducing the time and costs of the analysis and leading to better detection limits.

The aim of this work is to evaluate the use of cryogenic grinding technique as a rapid sample preparation method for copper, lead and manganese determinations in tooth slurries by GFAAS with a W–Rh coated platform.

Experimental

Apparatus

A PerkinElmer (Überlingen, Germany), Model 4100ZL atomic absorption spectrometer with a longitudinal Zeeman-effect background correction system and a transversely heated graphite atomizer (THGA™) was used throughout. Slurries were sonicated by using a Vibracell VC 50-1 ultrasonic processor (50 W) with a titanium probe (Sonics & Materials, Danbury, CT, USA) controlled by a PerkinElmer USS-100 ultrasonic slurry sampler. Sample aliquots (20 μL) were delivered over the W–Rh coated platform of the transversally heated graphite atomizer (THGA™) tube with an AS-71 autosampler. Standard THGA™ graphite tubes with integrated platform, PerkinElmer (Part no. B050-4033), were used after thermal coating first with 250 μg W and then with 200 μg Rh (referred to as the W–Rh coated platform) as described elsewhere.[34]

Table 1 Heating programs for copper, lead and manganese determinations in tooth slurries and digests of tooth samples

Step	Temperature/°C	Ramp/s	Hold/s	Ar flow rate/mL min^{-1}
1	150	5	20	250
2	200	5	15	250
3	1200[a], 800[b], 1100[c]	10	20	250
4	2100[a], 1700[b], 2200[c]	0	5	0
5	2400	1	3	250

[a]For copper. [b]For lead. [c]For manganese.

All measurements were based on integrated absorbance and performed at 283.3 nm (slit 0.7 nm) for Pb by using an electrodeless discharge lamp (EDL-II system, PerkinElmer), at 324.8 nm (slit 0.7 nm) for Cu and 279.5 nm (slit 0.2 nm) for Mn by using PerkinElmer hollow cathode lamps. The heating programs used for this instrument for copper, manganese and lead determination in tooth slurries are given in Table 1, along with instrumental settings. Argon 99.96% was used as the protective gas throughout.

Tooth samples were ground with a cryogenic mill Model 6800 Freezer/mill (SPEX, Metuchen, NJ, USA). A laser particle size analyzer Model Mastersizer E (Malvern, Worcestershire, UK) was used for particle size measurements.

For sample decomposition a closed vessel microwave oven, ETHOS 1600 (Milestone, Sorisole, Italy) was used. **Note.** According to the Ethos 1600 manufacturer (Milestone), the minimum volume to ensure appropriate temperature control with a standard thermocouple sensor is 8 ml. There is a risk of damaging the digestion vessel if the sensor is not properly dipped into the liquid phase.

Reagents, materials and solutions

High purity de-ionized water (resistivity 18.2 mΩ cm) obtained by a Milli-Q® water purification system (Millipore, Bedford, MA, USA) was used throughout. Analytical reagent grade HNO_3 (Merck, Rio de Janeiro, Brazil) was distilled in quartz sub-boiling stills (Kürner, Rosenheim, Germany). All solutions were stored in polyethylene bottles. Plastic bottles, autosampler cups and glassware were cleaned by soaking in 10% v/v HNO_3 for 24 h, rinsing five times with Milli-Q® water and dried and stored in a class 100 laminar flow hood. The washing water used to clean the sampling capillary of the 4100 ZL equipment was replaced by a 0.2% v/v HNO_3 + 0.04% v/v Triton® X-100 (Amershaw, Searle, Arlington Heights, VA, USA) solution to avoid the autosampler pipette clogging.

Stock standard solutions containing 1000 mg L^{-1} of Cu, Pb and Mn were prepared by dissolution of appropriate masses of metallic Cu, $Pb(NO_3)_2$ and MnO_2 (Johnson Matthey, Royston, UK) in dilute HNO_3. Analytical calibration solutions were prepared by suitable dilution of stock solutions in 0.2% v/v HNO_3 + 0.04% v/v Triton® X-100.

Preparation of the carbide-forming chemical modifier (1.0 g L^{-1} W) and rhodium solution (1.0 g L^{-1} Rh) for graphite surface treatment is described elsewhere.[34]

The conventional chemical modifier (added at each firing) used with untreated pyrolytic graphite platforms for comparative purposes was 0.05% (m/v) Pd + 0.03% (m/v) $Mg(NO_3)_2$ prepared from 10.0 g L^{-1} Pd and 10.0 g L^{-1} $Mg(NO_3)_2$ solution (Suprapur®, Merck, Darmstadt, Germany).

Samples

A total of 12 human tooth samples were collected from children and adults by local dentists from São Paulo state, Brazil. Detailed information of each tooth was not obtained but only teeth without fillings were used. To remove residues of soft tissues before grinding, teeth were washed with 30% v/v H_2O_2

during 2 h with occasional agitation as described elsewhere.[9] After removing the soft tissue, whole teeth were washed with Milli-Q® water, dried in a class 100 laminar flow hood and oven-dried for 1 h at 105 °C.

Cryogenic tooth grinding

Cryogenic tooth grinding was accomplished by using a cryogenic mill with a self-contained liquid nitrogen bath (SPEX Model 6800 Freezer Mill). The equipment carries out the grinding by impact with magnetic bars. The cylindrical grinding vial consists of a polycarbonate center tube, a magnetic bar and two end plugs of stainless steel. The magnetic bar was introduced into the polycarbonate tube with a tooth sample and the polycarbonate tube was closed with steel end plugs. Each sample was placed in a separate grinding vial, which was self-contained and could be cleaned after each use, thus eliminating cross-sample contamination. A pre-cooling time of 5 min was adopted, followed by 2 min of grinding for deciduous tooth and 3 cycles of 2 min for permanent tooth samples.

Slurry preparation

Slurries were prepared directly in the autosampler cups by transferring an exact amount of homogeneous powdered material (20 mg) to the PTFE autosampler cups, followed by addition of 1.00 mL of solution containing 0.2% v/v HNO_3 and 0.04% v/v Triton® X-100. Slurries were sonicated with a titanium ultrasound probe for 20 s before delivering 20 μL into the platform previously coated with 250 μg W + 200 μg Rh. The heating programs shown in Table 1 were employed throughout for Cu, Mn and Pb determinations by GFAAS. Calibration were performed against standard aqueous solutions containing 0.2% v/v HNO_3 and 0.04% v/v Triton® X-100. Standard addition calibration was used for the comparative method with Pd/Mg as chemical modifier.

Wet digestion

For comparative purposes, 12 human tooth samples were also digested using a microwave-assisted method in closed vessels (Milestone, Ethos 1600). Teeth were decomposed in triplicate according to the following procedure: 100 mg of dried and ground material were accurately weighed in TFM® vessels of the microwave oven and then 2.0 mL of 20% v/v HNO_3 were added. After decomposition, the TFM® microwave vessels were cooled, the digestate transferred to 10 mL volumetric flasks and the volume adjusted with Milli-Q® water. The microwave operational parameters applied are given in Table 2 (maximum external vessel temperature at 90 °C).

Results and discussion

Cryogenic grinding

Deciduous and permanent teeth were transferred to the polycarbonate tube and the efficiency of the proposed cryogenic grinding technique was evaluated by applying arbitrarily a pre-cooling time of 5 min followed by 2 min grinding. For deciduous teeth, only 2 min of grinding was necessary to achieve a fine and totally ground powder. However, for permanent teeth 3 grinding cycles of 2 min were needed for obtaining a more homogeneous and smaller particle size distribution. A re-cooling step of 1 min was applied between each grinding cycle to avoid heating of both sample and grinding set. It is important to say that the 6800 Freezer Mill grinds single samples up to 100 g with the 6801 vial or four samples up to 2 g with 6751 vials simultaneously. Due to the size of some permanent teeth, the samples should be broken into small pieces before introduction into the 6751 vials.

Table 2 Microwave heating program for tooth digestion

Step	Time/min	Temperature/°C	Power/W
1	3	160	1000
2	2	160	0
3	5	230	1000
4	10	230	1000

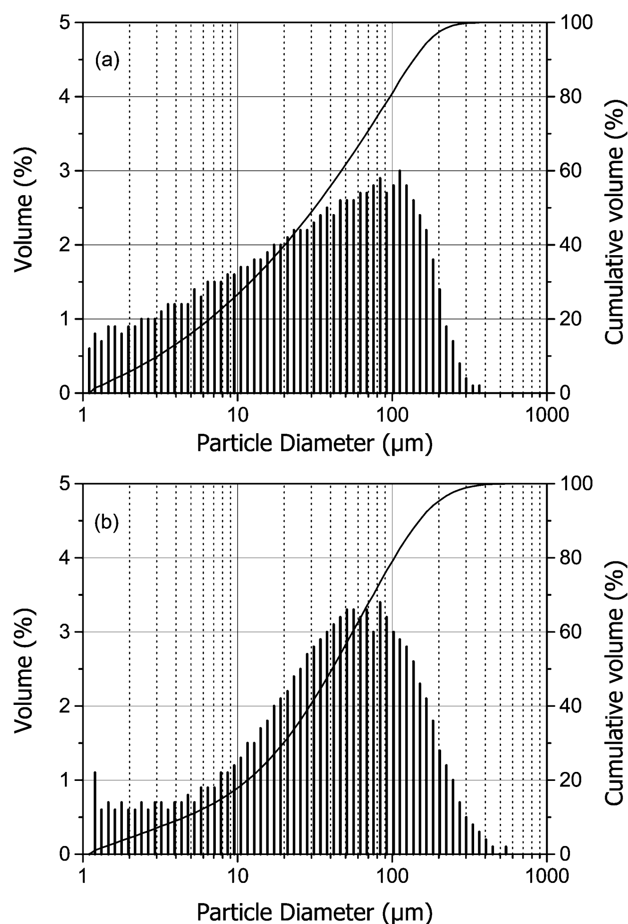

Fig. 1 Particle size distribution as relative particle volume *versus* size after cryogenic grinding: (a) 5 min of pre-cooling and 2 min of grinding of deciduous tooth; and (b) 5 min of pre-cooling and 3 × 2 min of grinding of permanent tooth.

Figs. 1(a) and 1(b) illustrate the particle size distributions for both ground teeth as relative particle volume *versus* size, after the first cryogenic grinding process, for deciduous and permanent tooth samples, respectively. As a result, 90% of total volume presented a homogeneous distribution composed by particles lower than 150 μm. The particle diameter medians of both samples were about 40 μm.

It can be pointed out that in most grinding techniques often used for sample preparation in atomic spectrometry, only one sample can be ground at time and the sampler container needs to be rigorously cleaned before the introduction of the next sample to avoid cross-sample contamination. These procedures decrease severely the sample throughput in routine analysis.

Pyrolysis curves

Pyrolysis temperature curves for Cu, Mn and Pb in aqueous solution and in tooth slurries with and without W–Rh coated platforms are shown in Fig. 2.

It can be observed (Fig. 2(a)) that copper is thermally stable up to 1200 °C in tooth slurries with W–Rh permanent chemical modifier and 1000 °C without modifier. In aqueous solution the permanent modifier increased the maximum pyrolysis temperature for copper from 900 to 1200 °C, which is in agreement

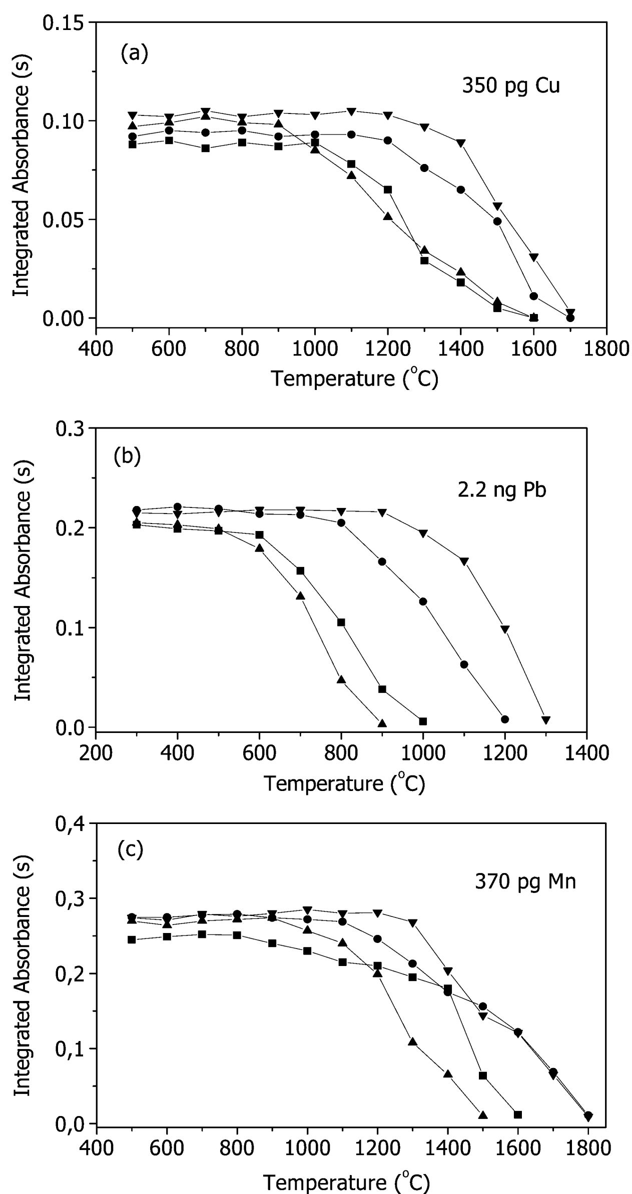

Fig. 2 Pyrolysis curves for (a) copper, (b) lead and (c) manganese. (■) Tooth slurry without modifier, (●) tooth slurry with W–Rh modifier, (▲) aqueous solution without modifier and (▼) aqueous solution with W–Rh modifier.

with the previous publication for copper determination in sediment slurries.[35] It can be pointed out that copper determination in tooth slurries could be made without the use of modifier by applying a maximum pyrolysis temperature of 900 °C. In this condition the relative standard deviation (RSD) of 3 replicates was generally $>9\%$ and the recovery was around 90%. However, when copper was determined in the same slurry samples by GFAAS using a W–Rh coated THGA platform, better RSD ($<4\%$) and recoveries (97%) were obtained.

Lead is a volatile element that is generally lost from the THGA tubes at temperatures higher than 500 °C in the absence of chemical modifiers. The most common modifier used for lead determination in clinical samples is $NH_4H_2PO_4$. According to Zong *et al.*,[41] who investigated the effects of bone matrix on Pb atomization, lead is lost from bone digestate even at very low pyrolysis temperatures (<600 °C) using the bone matrix as an endogenous source of phosphate. When 20 µg of $NH_4H_2PO_4$ was co-injected with the digestate as a chemical modifier, the pyrolysis step could be carried out at 900 °C, and authors[41] concluded that endogenous phosphate in bone was unavailable for lead chemical modification. As a consequence, the use of a modifier was recommended for lead determination in tooth samples. Spěváčková *et al.*[42] proposed a similar method for lead in acid digested deciduous teeth by GFAAS using a PE 4100 ZL and $NH_4H_2PO_4$ as a chemical modifier, and also evaluated the analyte stabilization of lead by endogenous phosphate. They observed that without the addition of an external source of phosphate, the analyte was stable up to 750 °C, and when a solution containing $NH_4H_2PO_4$ conventional chemical modifier was used, the maximum pyrolysis temperature was extended up to 950 °C.

In the present work, lead pyrolysis temperature curves (Fig. 2(b)) showed lead thermal stability up to 800 °C in tooth slurries (RSD $< 4.0\%$, $n = 3$) and 900 °C in aqueous solution (RSD $< 2.0\%$, $n = 3$), with W–Rh permanent chemical modifier. However, without W–Rh, the maximum pyrolysis temperature for lead decreased to 600 and 500 °C for slurries and aqueous solution, respectively, and the RSD for 3 replicates was higher than 10% in both media, indicating that the bone matrix did not remarkably improve Pb stabilization and that a graphite coated platform with 250 µg W plus 200 µg Rh is equally good for chemical modification. In addition, no overcorrection for lead was observed using the proposed method with the optimized heating program shown in Table 1. It must be mentioned that Zong *et al.*[41] found a background overcorrection for lead determination in bone-digested samples using $NH_4H_2PO_4$ modifier and a PE 4100 ZL equipped with longitudinal Zeeman-effect background correction. On the other hand, authors reported successful determinations of lead in bone using $NH_4H_2PO_4$ as a chemical modifier when using a spectrometer from the same manufacturer equipped with transverse Zeeman-effect background correction (PE Z5100) and a Massmann-type longitudinally heated HGA-600 graphite furnace.

For manganese in slurries and in aqueous solution, good thermal stabilization was also observed when W–Rh permanent chemical modifier was used. Maximum pyrolysis temperature increased from 800 °C without modifier to 1100 °C with W–Rh modifier in tooth slurries, and from 900 to 1200 °C in aqueous solution (Fig. 2(c)). Although the stabilization of manganese in tooth slurries was attained up to 800 °C without modifier, manganese recoveries were lower than 83% with RSD $>10\%$ ($n = 3$) for all tooth samples tested, probably as a result of analyte occlusion in the matrix particles.[33] However, with the W–Rh permanent modifier and pyrolysis at 1100 °C, quantitative recoveries were attained for all the same samples with RSD $< 5\%$ ($n = 3$).

Typical atomization profiles for 300 pg Cu, 90 pg Mn and 700 pg Pb in aqueous solution and in 2.0% m/v tooth slurries are shown in Fig. 3. For this study, sample aliquots (20 µL) were delivered into the previously W–Rh coated THGA platform. Small differences in peak profiles can be observed for manganese and copper in both aqueous solution and slurries, but they were not relevant for method implementation.

Slurry optimization

The success of the slurry sampling technique depends generally on the sample particle diameter, sub-sample homogeneity, suspension medium, stirring method and sampling depth. In this work all of these parameters were taken into account in order to achieve better accuracy and precision.

After grinding, tooth suspensions were prepared directly in the autosampler cups with 1.00 mL of 0.2% v/v HNO_3 + 0.04% m/v Triton® X-100. The sampling depth exploited by the autosampler PTFE capillary in the slurry was kept at 10 mm to avoid sedimentation errors, as recommended.[25]

Usually, the influence of sample inhomogeneity on precision and accuracy is greater for slurries containing less than 0.2% m/v of sample, because small numbers of particles are introduced into the atomizer.[43] When working with concentrated slurries

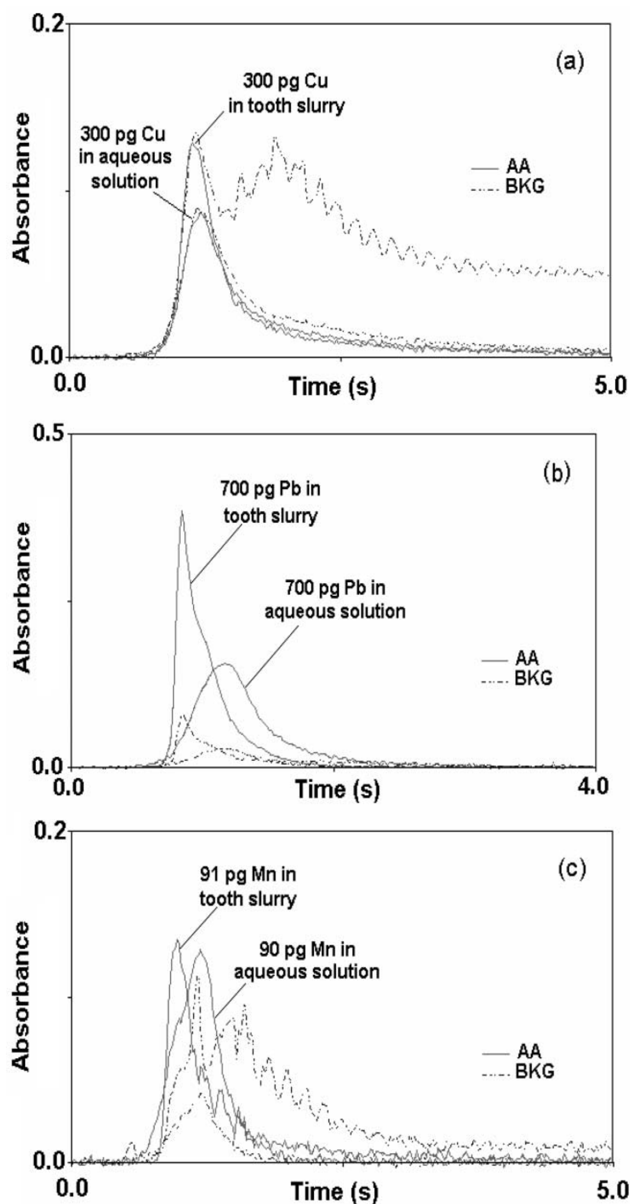

Fig. 3 Typical atomization peak profiles for (a) copper, (b) lead and (c) manganese in tooth slurry and aqueous solution from a THGA® tube with W–Rh coated platform using the heating programs shown in Table 1.

the relationship of sample introduced and absorbance can be affected by the content of analyte in the sample if the rollover absorbance region is reached. In addition, at higher slurry concentrations, the amount of interferents introduced is increased and systematic errors are expected.[38] In order to optimize the slurry concentration, the experiments were performed within 0.5 and 3.0% m/v tooth slurries, using W–Rh permanent modifier. For slurries containing up to 3.0% m/v, there was a linear relationship between integrated absorbance and amount of sample introduced into the atomizer. However, the introduction of large amounts of sample into the graphite tube contributed to the high build-up of inorganic residue on the platform surface after 100 atomization cycles, impairing the quality of the results. The inorganic residues formed probably decrease the interaction of analyte with the permanent modifier onto the coated platform. Taking into account the mean copper, manganese and lead contents in the samples and the precision of measurements, it was decided to work with tooth slurries up to 2.0% m/v and cleaning temperatures of 2600 °C (graphite tube wall temperature) to minimize the build-up of inorganic residues.

Sub-sample homogeneity

Trace elements tend to be inhomogeneously distributed throughout bone or tooth samples.[16] However, for monitoring proposes, it is very important to guarantee both sample homogenization and sample representativeness. Generally, homogenization is critical and samples must be ground to a fine powder. In the slurry sampling technique all sampling operations have to be performed with care in order to obtain a representative sample and to reduce sampling uncertainty to a minimum.[44] Minor components have less homogeneity in teeth than in other inorganic solid samples, as they occur in hydroxyapatite and/or enamel protein and collagen.[16] Therefore, they must be ground to produce a suitable particle size distribution to make them homogeneous for better reproducibility and representativeness. In this way, Nakamura *et al.*[16] showed that samples must be ground to particles smaller than 10 μm for determination of cadmium and lead in human teeth by direct solid sampling in graphite furnace atomic absorption spectrometry (SS-GFAAS).

Miller-Ihli[24] has pointed out that due to the small amount of sample atomized by the slurry technique (*e.g.*, 20 μL of a 1% m/v slurry represents 200 μg delivered into the atomizer), slurry sampling in GFAAS is well suited to characterization of the homogeneity of solid sample at the microgram or milligram level, depending on the analyte extraction into the liquid phase. In the case of 0% extraction the between-batch precision is mainly determined by the homogeneity of the mass delivered into the atomizer. When the analyte extraction is 100% the between-batch precision is determined by the homogeneity of the whole mass used for slurry preparation and designated representative sample mass, which can be calculated according to Miller-Ihli.[45]

It is important to point out that for 1% m/v slurries prepared directly in autosampler cups by addition of 10 mg of the ground sample in 1 mL of diluent, the representative mass of 200 μg in 20 μL delivered over the graphite platform is increased to 10 mg when 100% of analyte is extracted in the liquid phase. Therefore, it is important to stress that the content of analyte extracted in liquid phase will probably contribute to the success of the slurry sampling technique mainly for samples with known inhomogeneity in analyte distribution.

However, as described by Santos *et al.*[28] the extraction efficiency is generally dependent on sample characteristics, on stirring method and on sample mass used for slurry preparation. When 2% m/v tooth slurries in 0.2% v/v HNO_3 and 0.04% v/v Triton® X-100 were sonicated during 20 s, the fraction of analyte extracted in liquid phase was not constant, varying from 14 to 22%, from 17 to 33% and from 28 to 37%, for copper, manganese and lead, respectively. Thus, knowledge of the minimum mass necessary to prepare the slurry and obtain acceptable accuracy and precision is crucial. In this way, the precision dependence in sub-samples related to the amount of powdered material (5–20 mg) was investigated. From deciduous ground tooth samples arbitrarily chosen, 10 slurries were prepared for each sub-sample by transferring 5.0, 10.0 or 20.0 mg of powdered materials to autosampler cups, followed by the addition of 1 mL of diluent solution. All measurements were made with three replicates (within-batch) and the results were compared with the wet-digested (100.0 mg) method described herewith.

By applying an analysis of variance (ANOVA), the minimum masses necessary for slurry preparation were 20 mg for lead and manganese and 10 mg for copper determination. The mass level indicated could not be used to guarantee the homogeneity of ground samples. However, between-batch precision ($<6\%$) and within-batch precision ($<4\%$), as well as the fact that no significant differences were found at 95% confidence level in comparison with analyte concentration after wet digestion, indicate that acceptable accuracy and precision for slurry

Table 3 Copper, lead and manganese determination in slurries and digests of tooth by GFAAS

CRM	Cu/μg g^{-1}		Pb/μg g^{-1}		Mn/μg g^{-1}	
	Digest[b] (n = 3)	Slurry (n = 3)[a]	Certified (n = 3)	Slurry (n = 3)[a]	Digest[b] (n = 3)	Slurry (n = 3)[a]
Animal Bone (IAEA-H5)[c]	0.62 ± 0.01	0.69 ± 0.03	3.10 ± 0.56	3.30 ± 0.12	0.80 ± 0.04	0.82 ± 0.04
Samples	Digest[b] (n = 3)	Slurry (n = 3)[a]	Digest[b] (n = 3)	Slurry (n = 3)[a]	Digest[b] (n = 3)	Slurry (n = 3)[a]
1	5.04 ± 0.41	5.26 ± 0.36	1.53 ± 0.07	1.44 ± 0.09	0.72 ± 0.03	0.76 ± 0.03
2	5.11 ± 0.32	4.77 ± 0.22	1.18 ± 0.05	1.05 ± 0.06	0.81 ± 0.07	0.85 ± 0.05
3	1.03 ± 0.05	1.18 ± 0.08	9.72 ± 0.31	10.25 ± 0.89	0.77 ± 0.06	0.72 ± 0.07
4	0.95 ± 0.04	0.87 ± 0.07	12.64 ± 1.04	11.70 ± 0.72	0.64 ± 0.07	0.68 ± 0.03
5	1.39 ± 0.07	1.27 ± 0.03	3.25 ± 0.22	3.40 ± 0.14	1.71 ± 0.15	1.62 ± 0.07
6	0.84 ± 0.09	0.79 ± 0.06	3.53 ± 0.20	3.71 ± 0.35	0.57 ± 0.07	0.61 ± 0.03
7	1.25 ± 0.11	1.38 ± 0.06	13.91± 0.68	14.25 ± 0.47	1.45 ± 0.09	1.53 ± 0.13
8	2.34 ± 0.16	2.23 ± 0.07	8.16 ± 0.62	7.23 ± 0.35	1.05 ± 0.06	1.15 ± 0.09
9	0.95 ± 0.09	0.89 ± 0.07	13.41 ± 1.14	12.52 ± 0.69	0.59 ± 0.03	0.63 ± 0.07
10	2.45 ± 0.08	2.33 ± 0.01	12.44 ± 0.69	13.37 ± 1.01	2.23 ± 0.17	2.46 ± 0.21
11	1.85 ± 0.13	1.76 ± 0.09	6.94 ± 0.55	6.48 ± 0.25	0.67 ± 0.04	0.74 ± 0.02
12	2.62 ± 0.21	2.53 ± 0.18	2.61 ± 0.22	2.82 ± 0.17	0.83 ± 0.08	0.72 ± 0.06

[a]Three slurries were prepared directly in autosampler cups with three measurements for each cup. [b]Three digests were prepared by the aforementioned method and three measurements were made for each digest. [c]No certified reference values are available for copper and manganese.

preparation were achieved. In addition, there were no significant differences at the 95% confidence level when the cryogenic grinding program was varied from 1 to 6 cycles of 2 min for deciduous teeth.

Analytical characteristics

The proposed slurry sampling method uses analytical calibration with aqueous standards for lead, copper and manganese. The characteristic masses obtained based on integrated absorbances were 30 pg Pb, 16 pg Cu and 6.0 pg Mn with standard THGA tubes. Method detection limits, for 2.0% m/v tooth slurries, were 34.0 ng g^{-1} Pb, 18.0 ng g^{-1} Cu and 7.4 ng g^{-1} Mn, employing W–Rh permanent chemical modifier.

The use of the W–Rh coated platform increased the tube lifetime for Cu, Pb and Mn determination in tooth slurries, leading to a remarkable decrease in the variable analytical costs, similar to those already estimated.[40] The W–Rh coating remained stable for approximately 300 analytical firings when 20 μL of 2.0% m/v tooth slurries were delivered onto the treated platform.

Method validation and sample analysis

For method validation, a bone certified reference material (H-5 Animal Bone) from the IAEA was analyzed. There is no certified reference material available on the market for bone or teeth with certified contents for copper and manganese, so the influence of the matrix on copper and manganese determinations in tooth slurries was evaluated by comparing the results of 12 tooth samples analyzed by the proposed slurry sampling method and by a Pd/Mg procedure with the same samples acid digested with HNO_3 and using the analyte addition method (Table 3). The result found for lead in CRM H-5 Animal Bone showed a good agreement with the certified value. Also, good agreement was observed when comparing the results for copper, lead and manganese determination in 12 human samples and in the H-5 animal bone material, using the proposed method and the comparative Pd/Mg method, with no statistical differences at 95% confidence level after applying the paired *t*-test.

Conclusion

Tooth analysis could be used as an alternative to traditional invasive methods for metals screening in child populations. In this way, fast sample analysis methods would minimize sample manipulation and contamination, as well as the cost of a routine analysis. Cryogenic sample grinding and slurry sampling were found to be an appropriate method for lead, manganese and copper determinations in teeth by GFAAS. It was demonstrated that using only 5 min of pre-cooling and 2 min grinding, it was possible to obtain homogeneous particle size distribution for deciduous teeth with 90% particle diameters generally lower than 150 μm. Homogeneity tests of different samples demonstrated that sample masses of 20 mg of cryogenically ground materials can be directly transferred to autosampler cups for ultrasound assisted slurry sampling, with further direct introduction of tooth slurry into the graphite atomizer of an atomic absorption spectrometer. Method analytical characteristics (*i.e.*, repeatability, reproducibility and representativity) presented above, using sub-sample masses of 10 mg for Cu and 20 mg for Pb and Mn, and tooth particle size distribution ($<$150 μm) obtained after cryogenic grinding for slurry preparation demonstrated a much greater tolerance of large particle size distribution using slurry sampling GFAAS when compared with direct sampling in a graphite furnace (SS-GFAAS).[16]

The lack of a suitable tooth reference material was not critical for analytical calibration in the slurry sampling proposed method, since aqueous standards can be used for all of the tested analytes. It is clear that for better analytical quality control more appropriate reference materials should be developed, and the proposed method will certainly be useful for certifying Cu, Mn and Pb in teeth and bone.

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