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Determination of Cd and Pb in biological reference materials by electrothermal atomic absorption spectrometry: A comparison of three ultrasonic-based sample treatment procedures

C. Maduro [b], G. Vale [a], S. Alves [b], M. Galesio [b], M.D.R. Gomes da Silva [a], C. Fernandez [c], S. Catarino [d], M.G. Rivas [a], A.M. Mota [b], J.L. Capelo [a,*]

[a] *REQUIMTE, Departamento de Química Faculdade de Ciências e Tecnología, Universidade Nova de Lisboa, Quinta da Torre, 2829-516 Monte de Caparica, Portugal*
[b] *Centro de Química Estrutural, Instituto Superior Técnico de Lisboa, Avda Rovisco Pais, s/n, 1049-001 Lisboa, Portugal*
[c] *Laboratório de Análises do IST, Instituto Superior Técnico de Lisboa, Avda Rovisco Pais, s/n, 1049-001 Lisboa, Portugal*
[d] *INIAP, Estação Vitivinícola Nacional, 2565-191 Dois Portos, Portugal*

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Abstract

Three different ultrasonic-based sample treatment approaches, the automated ultrasonic slurry sampling, the ultrasonic assisted acid solid–liquid extraction (ASLE) and the enzymatic probe sonication (EPS) were compared and discussed for the determination of Cd and Pb by ET–AAS in biological reference materials. The sample mass chosen to perform the analysis was 10 mg and the liquid volume was 1 ml of nitric acid 1 M. The best results were obtained with the slurry procedure with which it was possible accurate and precise determination of the Cd and Pb content in four of the five reference materials studied. Optimum performance (total metal extraction) of ASLE assisted by ultrasound for Cd was only achieved in two of the four materials assessed whereas total Pb recovery was only possible in three of the five samples. Total extraction with the enzymatic probe sonication was only obtained for Cd in oyster tissue. Neither ASLE nor EPS were able to extract Cd or Pb from spruce needles. Pb concentration obtained after EPS was found to be highly dependent from sample centrifugation speed and time.

Keywords: Acid leaching; Ultrasonic treatment; Microwave digestion; ET–AAS; Enzymatic probe sonication

1. Introduction

Analysis of metals and metalloids in solid biological samples by atomic absorption spectrometry (AAS), atomic emission spectrometry (AES) or inductively coupled plasma mass spectrometry commonly requires the total dissolution of the sample to avoid the adversary effects on the analysis of the matrix in which the metal is present [1,2]. Although direct introduction of a solid for analysis of its metal content by spectrometry can be achieved in some instances, e.g. direct solid introduction in electrothermal atomization (ET)–AAS, such procedures are far from be reliable approaches for routine analysis laboratories. Progress in technology has improved metal dissolution from solid samples in such a way that for many metals and metalloids, total digestion of samples may be overcome by a simple solid–liquid extraction procedure. So far, recent trends in sample treatment for solid–liquid extraction of metals and metalloids includes the following items: (i) solid–liquid extraction assisted by ultrasonic probes or baths [3–5]; (ii) acid solid–liquid extraction (ASLE) assisted by microwave energy [6]; (iii) hot water

* Corresponding author. Tel.: +351 212949649; fax: +351 212949647.
E-mail address: jlcapelom@dq.fct.utl.pt (J.L. Capelo).

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Table 1
Performance of the three ultrasonic-based methods for the extraction of Pb and Cd compared in this work

Parameter	USLE	EPS	Slurry
Organic matter introduced into the graphite furnace	Low	Medium	High
Sonication power	>40%	10–40%	10–40%
Sonication time	5–10 min	<5 min	15–30 s
Liquid medium	5–10% (v/v) acid/s	Water/buffered	2–5% (v/v) acid
Representative sub-sample	Total sample weighed	Total sample weighed	Amount introduced into graphite
Sample size	Critical	Critical	Not critical
Sample throughput	High	High	Low
Species preservation	No	High	No
Sample centrifugation for cleaning purposes	Yes	No	No
Sample dilution	Yes	Yes	No

extraction [7]; (iv) pressurised liquid extraction [8]. Ultrasonic solid–liquid extraction (USLE) of metals from solid biological samples offers some advantages over other extraction/digestion methodologies, such as: (1) acid/s are used in lower extent; (2) fast extraction procedures, a sample can be ready in less than 30 s [9]; (3) high throughput, with the new multi-sonicators 12 samples can be treated at the same time [10]; (4) mild extraction conditions; (5) matching the analytical minimalism roles—(i) low solvent extraction volume, (ii) low sample mass and (iii) low sonication power.

USLE of metals and metalloids can be achieved by adding some chemicals to the liquid media in conjunction with ultrasonication. There are two different approaches for USLE depending on the analysis requirement: (i) the total metal or metalloid content is determined by using an acid in conjunction with sonication, usually nitric acid at low concentration, generally 1 M [3,4] and (ii) the metal or metalloids species present in a biological sample can be determined after their extraction using enzymes in conjunction with ultrasonication, enzyme probe sonication (EPS). EPS has been recently reported as a powerful tool for extracting metals preserving the integrity of their species in times as short as 30 s [9,11–13]. So far, USLE and EPS deserves more research, since it is far from be totally understood (i) the linking USLE (or EPS)–metal–matrix, i.e. for which metals and matrixes the procedures based on ultrasonication works well and (ii) the mechanism by which ultrasonic cavitation considerably boosts the enzyme–substrate kinetics in EPS. On the other hand, ultrasonication can be also utilized as a way to homogenize a finely pulverized solid sample in a liquid (slurry) previous its introduction into a graphite furnace. The ultrasonic-based slurry technique for the introduction of solid samples in ET–AAS was first reported by Epstein et al. [14] and nowadays is a well established technique for routine analysis. The analytical performance of the three ultrasonically based methods USLE, EPS and slurry are listed in Table 1.

The aim of the present work is to compare the three above mentioned approaches based on ultrasonic irradiation with probe for Pb and Cd determination in biological samples by ET–AAS. Five reference materials are used to assess the procedures described in the present work. In addition, microwave digestion (MWD) is used in those cases in which the metal content was not certified.

2. Experimental

2.1. Apparatus

A MLS 1200 mega microwave oven (Millestone, USA) was used for microwave digestion. A Branson Sonifier 150 ultrasonic cell disruptor–homogeniser (63 W, 22.5 kHz, Branson Ultrasonics Corporation, USA) equipped with a 3 mm titanium microtip was used. Ultrasonic energy irradiation was fixed at 10–40% level with the 2 mm microtip. The Sonifier 150 has a digital LCD display which provides a continuous read-out of the watts delivered to the end of the probe (range 5–6 W in this work). Eppendorf cups (2 ml) were used throughout this work. A Varian (Cambridge, UK) atomic absorption spectrometer model SpectrAA-300 plus equipped with a graphite furnace and an auto-sampler plus Zeeman background correction was used. Pyrolytic graphite-coated graphite tubes with L'Vov platform were used. The electrothermal parameters and thermal programmes are listed in Table 2.

Gel electrophoresis studies were performed with a Pharmacia LKB-GPS 200/400 (150 V, 15% SDS-PAGE), low molecular range weight standards from Amersham-Pharmacia (Uppsala, Sweden) were used.

Table 2
Instrumental parameters and thermal programmes for Pb and Cd

	Pb	Cd
Lamp current (mA)	12	10
Wavelength (nm)	217.0	288.8
Spectral bandpass (nm)	0.5	0.5
Thermal programmes		
Stage	Temperature (°C)/time (s)	
	Pb	Cd
Dry1	85/5	85/5
Dry2	95/40	95/40
Dry3	120/20	120/20
Dry4	300/20	300/20
Ashing1	800/20	800/20
Ashing2	800/10	800/10
Atomization1	2400/1.1[a]	1800/1.1[a]
Atomization2	2400/3.0[a]	1800/3.0[a]
Cleaning	2400/2.0	2400/2.0

[a] The purge gas flow rate (300 ml min^{-1}) was stopped.

2.2. Reagents

Milli-Q ultrapure water was used throughout. HNO_3 (N 30,709) was purchased from Merck (Darmstadt, Germany,). H_2O_2 (N 31,642) was purchased from Sigma–Aldrich (St. Louis, USA). Palladium nitrate atomic absorption modifier solution was purchased from Perkin-Elmer (N BO 190,635). A non-specific protease, *Streptomyces griseus* (protease XIV) from Sigma–Aldrich was used (P 5147). All stock standard solutions (Pb and Cd, 1000 $\mu g\,g^{-1}$, Merck) were stored in a refrigerator at 4 °C and protected from light. Working standard solutions were prepared just before use by appropriate dilution of the stock standard solution in HNO_3 (Merck) 1% (v/v).

2.3. Certified reference material

The reference materials used in this work were purchased from the Institute for Reference Materials and Measurements, BCR, Europe and from the International Atomic Energy Agency, IAEA and were: BCR-101, spruce needles; BCR-414 plankton; BCR-679 white cabbage, BCR-710 oyster tissue; IAEA-0392, algae.

2.4. Sample treatments

2.4.1. USLE

Samples were prepared by accurately weighing 10 mg into auto-sampler cups. One millilitre of ultra-pure water acidified with nitric acid 1 M was added to the solid. Sonication with a probe was applied for a certain time. The cup was placed in the auto-sampler and allowed to stand without further stirring.

2.4.2. EPS

Samples were prepared mixing 10 mg of the certified materials with 5 mg of the enzyme protease XIV. One millilitre of ultra-pure water was added and then the mixture was sonicated for a certain time. The cup was placed in the auto-sampler and allowed to stand without further stirring.

2.4.3. Slurry

Samples were prepared by accurately weighing 10 mg into the auto-sampler cups. One millilitre of ultra-pure water acidified with nitric acid was added to the solid. Sonication with a probe was applied for 10 s and then the cup was situated in the auto-sampler. The slurry was formed by manually operation of the ultrasonic probe 3–4 s (25% amplitude) before the auto-sampler withdrawn.

2.4.4. MWD

The Pd and Cd concentration was determined, when necessary, after microwave digestion. To ca. 50 mg of sample placed in the microwave reactor was added 3 ml of HNO_3 (concentrated) and 0.5 ml of H_2O_2 30% (v/v). Once the reactor was cupped, it was placed in the microwave oven and the following program was runned: step 1, S1, 1 min at 250 W; S2 3 min at 0 W; S3, 5 min at 250 W; S4, 5 min at 400 W; S5, 5 min at 600 W. After cooling to ambient temperature, the reactor was opened and the resultant solution was transferred into a 10 ml calibrated flask. The reactor was carefully cleaned with 3 ml of milli-Q water using a Pasteur pipette. Finally, the solution was made up to volume with milli-Q water.

3. Results and discussion

The thermal program, the matrix modifier, nitrate palladium and its amount were selected on basis on previous works [15,16]. Instead of a time-consuming optimisation of the thermal program, the amount of chemical modifier used (1000 $\mu g\,g^{-1}$, 10 μl) was tested against the slurry procedure, which introduces the maximum amount of organic matter into the graphite furnace from all of the procedures here assessed. It is well-known that the higher the organic matter content, the higher the possibilities to loose an element during the pyrolysis stage. In addition, other problems, such as chemical interferences due to incomplete organic matter destruction during the pyrolysis can arise during the atomization step. As can be seen in Table 3, the recoveries achieved for the certified materials with the slurry method did not statistically differ from their certified, recommend or MWD values, with the exception of Cd in white cabbage, for which the recovery was, however, higher that 90%. Thus, the amount of matrix modifier and the thermal programs were used without further modifications. Owing to the low Cd content of IAEA-0392,

Table 3
Pb and Cd concentrations obtained with the slurry method

Sample	Pb ($\mu g\,g^{-1}$, $n=4$)				Cd ($\mu g\,g^{-1}$, $n=4$)			
	Slurry value ($x \pm$ S.D.)	Certified value	R (%)	t_{exp}[a]	Slurry value ($x \pm$ S.D.)	Certified value	R (%)	t_{exp}[a]
BCR-101, spruce needles	–	2600	–	–	–	350	–	–
BCR-414, plankton	4.1 ± 0.3	3.97 ± 0.19	103	0.87	0.39 ± 0.04	0.383 ± 0.014	102	0.35
BCR-679, white cabbage	5.4 ± 0.5	5.9 ± 0.8[b]	92	0.43[c]	1.55 ± 0.03	1.66 ± 0.07	93	7.3
BCR-710, oyster tissue	4 ± 1	4.5 ± 0.3[b]	89	0.39[c]	0.97 ± 0.05	0.9 ± 0.1[b]	108	1.41[c]
IAEA-0392, algae	0.56 ± 0.03	$55.34E{-}2 \pm 45.30E{-}3$	101	0.44	–	$7.90E{-}3 \pm 22.55E{-}4$	–	–

[a] $t_{tab} = 3.18$ for $n = 4$, $P = 0.05$.
[b] Values determined after microwave digestion ($n = 4$).
[c] $t_{tab} = 2.45$ for $n = 6$, $P = 0.05$.

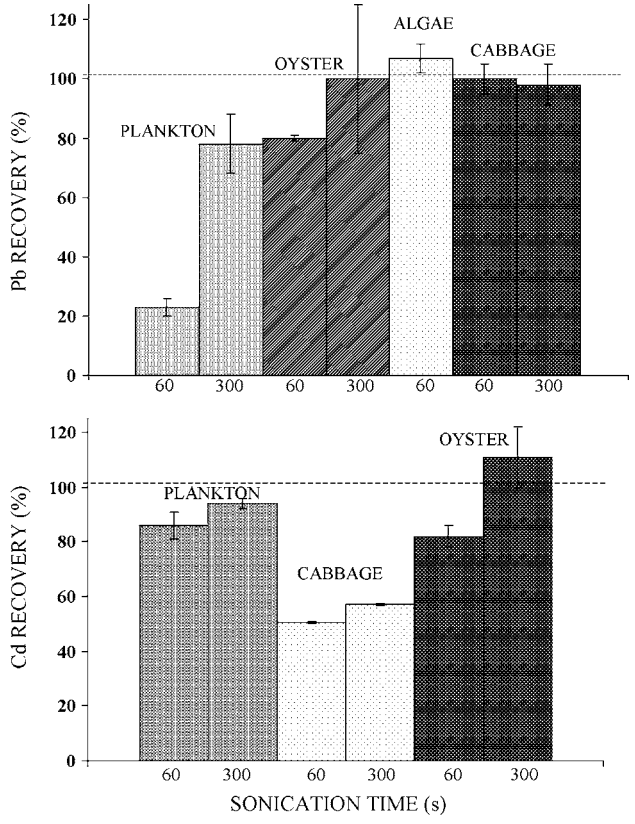

Fig. 1. Acid-USLE of Pb and Cd from reference material: 10 mg of sample mass plus 1 ml of 1 M HNO_3. Sonication amplitude 50%; output wattage 9–10 W; probe diameter 3 mm.

algae, this metal was not determined in this material, since the sample amount chosen for our studies, 10 mg, was not enough to obtain a solution with content higher than our detection limit for this metal. In the case of material BCR-101, spruce needles, owing to the high Pb and Cd levels, a sample treatment slightly different was attempted: an ultrasonic extraction was first performed and then an aliquot of the extract (50 μl) was diluted (500 ml) to obtain a solution with metal concentrations in the range of the calibration graph. Dilution of the ultrasonic extracts avoids organic matter interferences; this is one advantage of the USLE methodology, when the total extraction is achieved, dilution is possible, facilitating the performance of the analysis [16,17] as follows: (i) the quantity of matrix modifier needed is diminished, (ii) the pyrolysis stage can be omitted and (iii) the build-up of the carbonaceous residues inside the tube is avoided.

3.1. Acid-USLE

Fig. 1 shows the extraction yields obtained after USLE in an acidic media, 1 M HNO_3, with two different sonication times 60 and 300 s for plankton, oyster, algae and cabbage matrices. As can be seen, Pb was easily extracted from algae, oyster and cabbage materials. In algae and cabbage matrices, a short sonication time was enough to extract total Pb into the liquid media, whereas 300 s was necessary in the case of the oyster tissue. In the latter case, however, with 60 s of ultrasonication, ca. 80% of the metal was extracted with an excellent 1% R.S.D. ($n = 4$). Total extraction in oyster was achieved after 300 s of sonication, but the RSD was very high, 25% ($n = 4$). This fact could be explained on basis on the "nugget" theory as proposed by Kurfürst [18], the final 20% of the lead extracted when the sonication time was 300 s possibly had a different tissue source (with a stronger Pb binding in this case) and was heterogeneously distributed in the oyster, at least for the working sample mass, 10 mg. In the case of plankton, 300 s of sonication extracted an amount of ca. 80% of the expected Pb. Higher sonication times with this sample were not tried. In the author's experience, sonication times with probe higher than 300 s should be avoided, because the mechanical stress induced to the probe quickly reduces the useful probe life leading to inconsistent results. In addition, what a sonication probe can extract in 300 s usually can also be extracted with a sonication bath just using longer treatments times. The drawback of longer sonication times (e.g. 30–60 min) is compensated by the number of samples that can be treated at once. In the case of the reference material spruce needles, the recoveries were unexpectedly low regardless of the sonication time used. The aforementioned second dilution used in order to dilute the expected high concentration for the extracts of this sample was unnecessary since the amount extracted after the acid ultrasonic treatment was less than ca. 0.1% of the certified content for cadmium and lead. The extraction yield obtained for cabbage was ca. 100% regardless the sonication time used.

In the case of cadmium (Fig. 1), total recovery was achieved for the oyster tissue, whilst more than 90% was extracted from plankton with the maximum sonication time, 300 s. For plankton, high yields were obtained, more than 80%, with the minimum treatment time, 60 s. In the case of cabbage, the improvement obtained when the sonication time was increased from 60 to 300 s was low, only 7%, being the total extraction achieved almost 60%. These recoveries obtained for Cd with the acid-USLE from cabbage are similar to the ones reported by Dowrowolski and Mierzwa [19] in the same matrix with a similar methodology, 30 s of sonication with probe in a 5% (v/v) HNO_3 solution.

3.2. EPS extraction

Fig. 2 shows the results obtained after sonication in the presence of the enzyme protease. In the case of cabbage and spruce needles, neither cadmium nor lead was extracted. This result is according with the matrixes and enzyme studied. Thus, proteases attack the peptides bonds of proteins and peptides themselves; hence, this kind of enzyme is not adequate to hydrolyze starch and glycogen, main components of cabbage and spruce needles [9]. As can be seen in Fig. 2, Cd

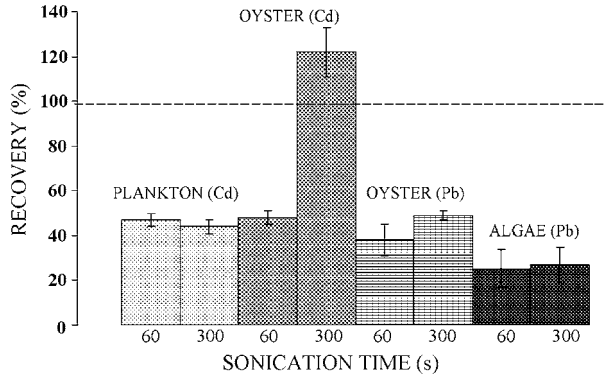

Fig. 2. EPS of Pb and Cd from reference material: 10 mg of sample mass plus 5 mg of protease XIV enzyme. No buffer was used. Sonication amplitude 50%; output wattage 9–10 W; probe diameter 3 mm.

was totally extracted from oyster tissue after 300 s of EPS, whereas an amount of 50% was recovered from plankton tissue using the same time. Interestingly, no improvement in the extraction efficiency was observed when the treatment time was increased from 60 to 300 s for this matrix. In the case of lead, low recoveries were achieved from oyster and algae, ca. 50 and 27%, respectively, with the highest sonication time, and no metal was found in the supernatant for plankton.

The fact that in some matrices, such as oyster and plankton, the performance achieved with EPS was better for Cd than for Pb allowed us to hypothesize that the macromolecules obtained as a result of the enzymatic treatment had different sizes, and that probably the Pb was associated with the protein fraction that quickly settle down after sonication. This hypothesis was confirmed by the centrifugation studies developed with oyster tissue to avoid the presence of large organic molecules in the supernatant in order to overcome the drawback of high organic contents in the liquid extract when working with techniques, such as ICP–OES, ICP–MS, CV–AAS, HG–AAS, CV–AFS or HG–AFS. The results of these studies are shown in Fig. 3. As can be seen, there is no change in the cadmium content of the supernatant when the sample was centrifuged at 3000 or 10,000 rpm during 1, 5 or 10 min. On the contrary, speed of centrifugation and centrifugation time severely affects the lead content of the liquid extract. Thus, it was stated that centrifugation should be avoided when working with EPS. Other consequence is the fact that when a metal is associated with the large molecular fraction, it can settle down whilst the sample is standing to be analysed.

Several studies were conducted in order to assess the effects of ultrasonication over the enzyme. Fig. 4 shows the gel electrophoresis of the enzyme protease XIV after different sonication times. As can be seen, there is a clear difference between the enzyme electrophoresis with or without sonication. Apparently, some degradation is observed, but results are inconclusive since the protein distributions are similar regardless the sonication time used (range 15–300 s).

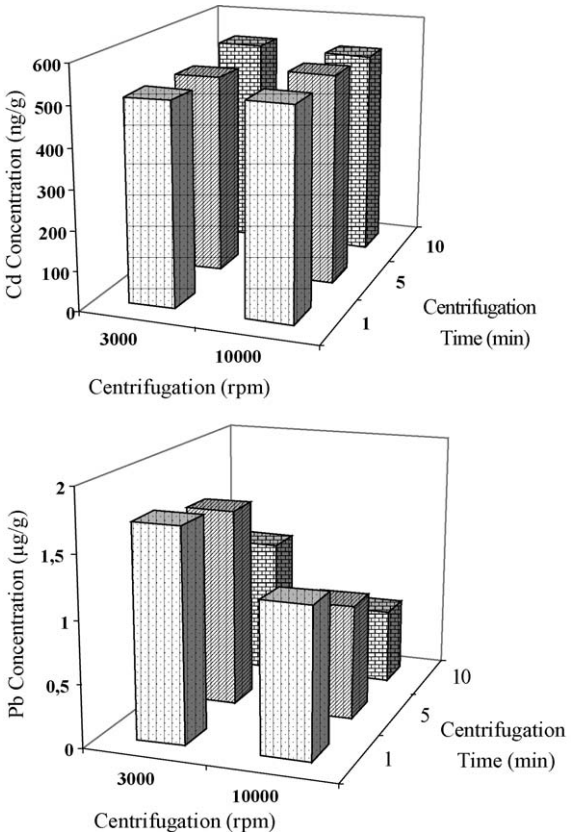

Fig. 3. Effect of centrifugation over metal concentration in the supernatant in EPS. EPS conditions were non-optimal: 20 mg oyster tissue; 5 mg protease XIV; sonication time 15 s; output wattage 4–5 W (10% nominal power); sonication volume 1 ml; Tris-buffer; pH 7.5.

3.3. Analytical figures of merit

Calibration was performed with a series of Cd and Pb standards. Sensitivity (m) was the slope value obtained by least-square regression analysis of calibration curves based on peak area measurements. The equation ($n = 5$) for the cal-

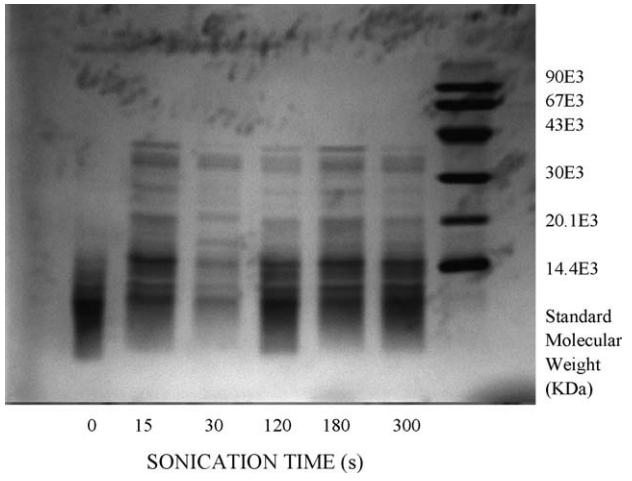

Fig. 4. Gel electrophoresis of protease XIV after sonication at different times.

ibration curves were as follows:

$$Y = (0.0011 \pm 2 \times 10^{-4})\,(\mathrm{Cd}) + (0.001 \pm 0.002)$$

$$Y = (0.00019 \pm 2 \times 10^{-4})\,(\mathrm{Pb}) + (0.0013 \pm 0.0004)$$

where Y is peak area, and Cd and Pb are the cadmium and lead masses deposited in the furnace in pg. The linear range of the calibration curve ranged from the quantification limit up to 10 $\mu g\,l^{-1}$ for Cd and up to 100 $\mu g\,l^{-1}$ for lead. The limit of detection (LOD), equal to 2 and 4 pg for Cd and Pb, respectively, was defined as $3sm^{-1}$, s being the standard deviation corresponding to 10 blank injections and m the slope of the calibration graph. The quantification limit (LOQ), defined as $10sm^{-1}$, was 7 and 14 pg, respectively, for Cd and Pb.

4. Conclusions

It was clearly stated from the three ultrasonic-based sample treatments studied in this work that the slurry methodology can be applied for trace metal determination to different sample matrixes, being less matrix dependent than the acid-USLE or EPS. Only with this methodology was possible to quantify the Cd and Pb contents in all the samples here studied with the exception of the spruce needles. In this case, due to the high levels for both metals, it was impossible their determination by this methodology. In cases like this, the possibility of dilution is of great help. For this reason, when (i) the sample has a high metal content and (ii) the acid-USLE or EPS methodology is not applicable owing to the lack in the extraction eficiency, the microwave digestion remains the best choice, since it allows dilution of the sample digest. Acid-USLE was only able to extract totally or almost totally (>90%) Cd and Pb content from soft tissues, such as plankton, algae and oyster tissues. From samples, such as cabbage or spruce needles, a fraction of the total content is extracted, but an important amount remains non-extractable. On basis in the author's experience and in literature [3,4,10], we recommend the slurry technique when working with plant material. EPS seems to be an approach uncompetitive when comparing it with the slurry technique or with the acid-USLE for total metal quantification; total extraction was only achieved in the case of Cd for oyster tissue whilst partial extraction of Pb was found in oyster and plankton. In addition, metal concentration for Pb obtained after EPS was found to be highly dependent from centrifugation speed and time. Although for total extraction, EPS seems not to be an alternative, this new methodology deserves more research, since its applications for speciation [10–13] are far from be well established. More research is actually developed in our lab in order to elucidate by which mechanism the focused ultrasound boots the kinetics substrate–enzyme, since the catalytic activity of the proteolytic enzyme was dramatically improved and not only in the kinetic terms of the extraction but also in the yield of the proteolytic activity.

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