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In memory of prof. dr. Simion Gocan

SIMULTANEOUS DETERMINATION OF Zn, Cd, Pb AND Cu IN MUSHROOMS BY DIFFERENTIAL PULSE ANODIC STRIPPING VOLTAMMETRY

ENIKŐ COVACI[a,*], EUGEN DARVASI[a], MICHAELA PONTA[a]

ABSTRACT. The present work presents the optimization of differential pulse anodic stripping voltammetry with hanging mercury drop electrode for the determination of Zn, Cu, Cd and Pb in mushrooms. The optimized method was characterized in terms of limits of detection and quantification, accuracy and precision and applied for the analysis of 4 food supplements (Chaga and Shiitake powders; Reishi tablets and capsules containing a Mixture of mushroom extracts) and a fresh mushroom (King bolete *(Boletus Edulis)*). The concentrations of Zn and Cu as essential elements were discussed in relation with the recommended daily allowance, while Cd and Pb compared with maximum acceptable levels of toxic elements set in the European legislation. It has been found that fresh mushroom King bolete (100 g serving per day) represents a more significant source of Cu and Zn than food supplements, namely up to 24% and 10% from the recommended daily allowance. The concentration of Cd (0.09-0.69 μg g^{-1}) and Pb (1.83-3.60 μg g^{-1}) in the edible King bolete fungus and food supplements revealed no human health risk, since they were below 1.5 mg provisional tolerable monthly intake (Cd) and 72 mg/day (Pb) for 60 kg body weight.

Keywords: *Differential pulse anodic stripping voltammetry, Zn, Cd, Pb, Cu, mushroom*

INTRODUCTION

Mushrooms and mushroom supplements are being consumed worldwide in a continuously increasing rate due to their countless therapeutic

[a] *Babeş-Bolyai University, Faculty of Chemistry and Chemical Engineering, 11 Arany Janos str., RO-400028, Cluj-Napoca, Romania.*
[*] *Corresponding author: kkeenniikkoo@yahoo.com*

effects, such as anticancer, antioxidant, immunostimulator and antihypercholesterolemic effects [1-8], although it has to be mentioned that so far no scientific evidence exists that proves these claims [9]. The most used medicinal mushrooms, like Reishi, Shiitake, Turkey tail, Chaga and Lion's mane, originate from China and Japan, where they have been used for centuries to treat many diseases [9]. Mushrooms, medicinal or edible, are known to be able to accumulate essential elements (e.g. Zn, Cu) and toxic heavy metals (e.g. Cd, Pb) [10], depending on species and environmental factors (soil composition, pollution) [11]. Some food supplements have had to be recalled because of noncompliance related to heavy metal content [12].

The importance of Cu and Zn determination is due to the fact that they are essential for the human organism as part of many essential proteins and enzymes with key roles in metabolic processes [13, 14]. The recommended values for daily allowance for adults set in Commission Regulation 2008/100/EC are 10 mg Zn and 1 mg Cu [15].

Cd and Pb determination are even more important because of their high toxicity and easy intake route, namely ingestion with contaminated foods. Cd negative effects include impaired immune and kidney functions [16], while Pb toxic effects cause damage of the central and peripheral nervous system, renal functions and vascular system [17].

Anodic stripping voltammetry is an attractive method for the determination of both essential and toxic metals in mushrooms with several advantages, such as the ability for simultaneous determination with good sensitivity, selectivity, precision and accuracy, possibility of element speciation and non-expensive instrumentation compared to other techniques such as graphite furnace atomic absorption spectrometry (GFAAS) [2,11] and inductively coupled plasma optical emission spectrometry (ICP-OES) [10,18,19]. Several researchers found differential pulse anodic stripping voltammetry (DPASV) with hanging mercury drop electrode (HMDE) suitable for the determination of Zn, Cd, Pb and Cu in a large variety of samples [20-27].

The aim of this study was quantification of Zn, Cd, Pb and Cu in mushrooms by DPASV with HMDE using the standard addition method. Prior determinations an optimization study was conducted regarding working conditions (deposition time, mercury drop size and concentration/volume of the solution used for standard additions) to achieve the best analytical performances. The method was characterized in terms of limit of detection, limit of quantification, precision and accuracy and applied for the analysis of mushroom food supplements (Reishi, Chaga, Shiitake and a Mixture of mushrooms extracts), and fresh mushroom (King bolete). The concentrations of Zn and Cu as essential elements found in samples were discussed in relation with the recommended daily allowance set in Commission Directive 2008/100/EC [15]. Cadmium and Pb were examined

in relation with maximum acceptable levels of toxic elements in mushrooms and mushroom supplements, set in Commission Regulation No 1881/2006 [28] and 629/2008 [29]. The risk of intoxication *via* ingestion of analyzed samples was examined in light of Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommendations [30].

RESULTS AND DISCUSSION

Method optimization

Prior to simultaneous determination of Zn, Cd, Pb and Cu by DPASV, several parameters such as volume/metal concentrations of the spiking solution, drop size and deposition time were optimized in order to achieve the best figures of merit for the method.

Multi-element standard solution

Optimization of the multi-element standard solution used in the standard addition calibration was considered necessary because of the uncertainty in precision of measurements when adding smaller volumes of higher concentration vs. higher volumes of lower concentration. For this, five multi-element standard solutions with variable metal concentrations were prepared, and four spikes were added to supporting electrolyte in each case (Table 1). The added amounts were adjusted to keep constant the final metal concentration in the electrolytic cell (59.8 μg L^{-1} Zn, 3.74 μg L^{-1} Cd, 3.12 μg L^{-1} Pb and 12.5 μg L^{-1} Cu). These metal concentrations were found to be appropriate to result in an increase of 2-3 times of the analytic signal in the standard addition method.

Table 1. Metal concentrations in the multi-standard solution and spike amounts for the optimization study

	Added volume (mL)	Metal concentration in the multi-element standard solution (mg L^{-1})			
		Zn	Cd	Pb	Cu
Standard solution 1	0.025	24.0	1.50	1.25	5.00
Standard solution 2	0.050	12.0	0.752	0.626	2.51
Standard solution 3	0.100	6.04	0.378	0.315	1.26
Standard solution 4	0.200	3.05	0.191	0.159	0.636
Standard solution 5	0.500	1.26	0.079	0.066	0.262

For each multi-element standard solution, the signal was plotted versus the metal ion concentration resulted in the electrolyte following successive spikes. The coefficients of determination (R^2) as statistical measure of linear relationship were calculated and plotted versus the added volumes (Fig. 1).

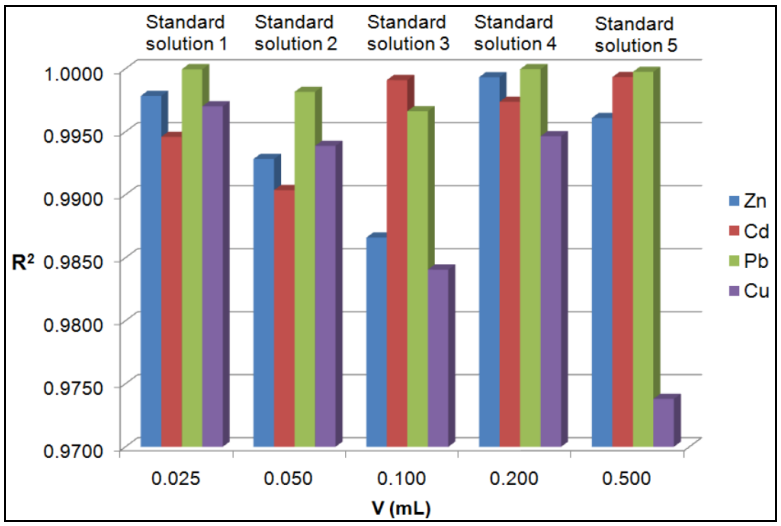

Figure 1. Effect of the volume/concentration of multi-element standard solution (Table 1) on the determination coefficient in standard addition in DPASV.

The spike volume of 0.200 ml (standard solution 4, Table 1) provided good coefficients of determination for all four metals (0.9993 for Zn, 0.9974 for Cd, 0.9999 for Pb and 0.9946 for Cu) and was selected as optimal for subsequent measurements. For few samples it was however necessary to adjust the concentration of the multi-element standard in order to keep constant the added volume of 0.2 mL.

Drop size

Mercury drop size needs to be optimized as it should provide suitable surface for an efficient deposition of metal ions. The optimal value was determined by varying the drop size, expressed in arbitrary units (a.u.) between 4-8 and recording the corresponding voltammograms (measuring conditions in Table 4) of a solution containing 10 mL supporting electrolyte and 0.2 mL multi-element standard solution 4. The signals for different drop sizes of HMDE (Fig. 2) indicated as optimal the drop size 6 (a.u.) as it provided the highest signals. All further experiments were carried out using this HMDE size.

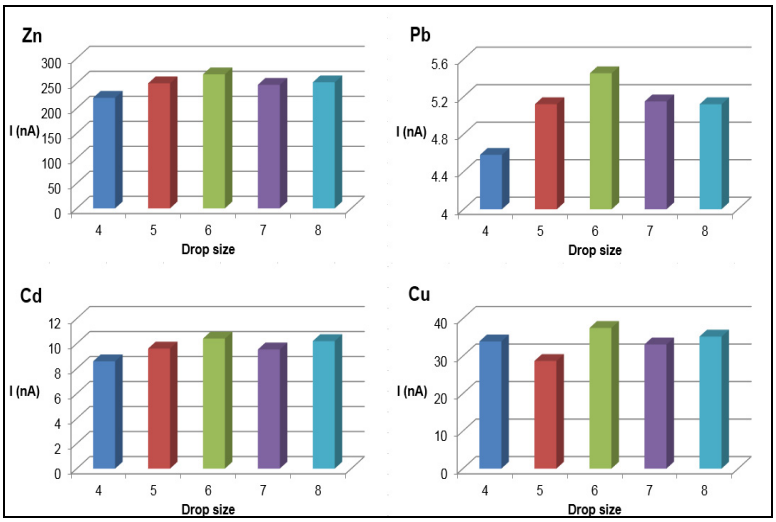

Figure 2. Effect of mercury drop size on peak height. Experimental conditions:10 mL electrolyte+0.2 mL multi-element standard solution 4 containing (mg L^{-1}): 3.05 Zn, 0.191 Cd, 0.159 Pb and 0.636 Cu.

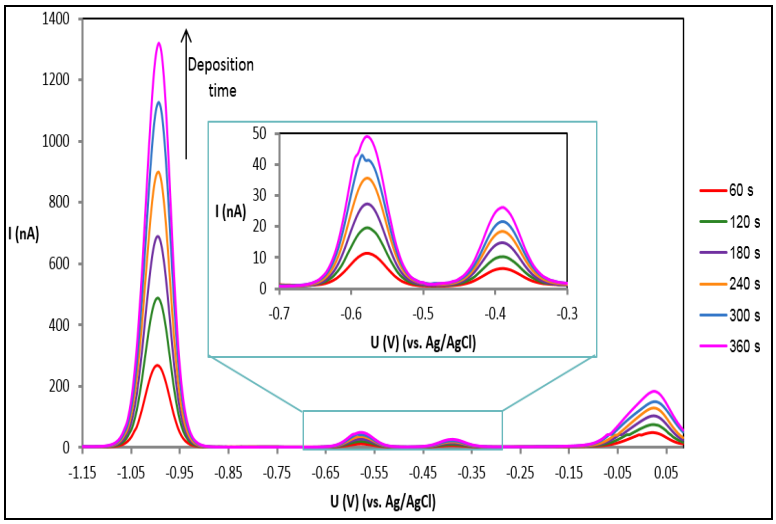

Figure 3. Effect of deposition time on peak height. Experimental conditions:10 mL electrolyte+0.2 mL multi-element standard solution 4 containing (mg L^{-1}): 3.05 Zn, 0.191 Cd, 0.159 Pb and 0.636 Cu.

Deposition time

The deposition time was optimized by recording the voltammogram of the multi-element standard solution using increasing deposition times in the range of 60-360 s with 60 s increments under the conditions given in the Experimental section (Table 5). For all four metals the peak height increased linearly with deposition time as a consequence of gradual metal concentration on the mercury drop (Fig. 3), however the use of long accumulation times renders analysis slow and impractical.

A deposition time of 60 s was found to be a good compromise between sensitivity and speed of analysis.

Method performances

Limits of detection and quantification

The DPASV method for the determination of Zn, Cd, Pb and Cu in mushrooms was characterized in terms of limit of detection (LoD) and limit of quantification (LoQ) in solution and dry mass under the optimized conditions. The LoD was calculated as $3s_{y/x}/b$, and LoQ as 3LoD, where $s_{y/x}$ is the residual standard deviation and b is the slope of the standard addition curve [31]. LoD and LoQ in solid samples were expressed taking into account the sample preparation protocol (Table 2).

Table 2. Limits of detection and quantification of Zn, Cd, Pb and Cu in mushrooms by DPASV

Metal	LoD[a] (μg L^{-1})	LoQ[b] (μg L^{-1})	LoD[c] (μg g^{-1} dry mass)	LoQ[c] (μg g^{-1} dry mass)
Zn	19.1	57.4	0.95	2.84
Cd	0.24	0.72	0.012	0.035
Pb	3.17	9.52	0.16	0.47
Cu	14.0	42.1	0.70	2.09

[a] calculated as $3s_{y/x}/b$.
[b] calculated as 3LoD.
[c] calculated for 1 g solid sample digested and diluted to 50 mL and 0.5 mL solution taken for analysis.

According to Table 2, lower LoDs were obtained for Cd (0.24 μg L^{-1}) and Pb (3.17 μg L^{-1}), and higher for Zn (19.1 μg L^{-1}) and Cu (14.0 μg L^{-1}). Compared to LoDs obtained in other reports for DPASV (0.26-0.69 μg L^{-1} Zn, 0.05-1.00 μg L^{-1} Cd, 0.5-0.8 μg L^{-1} Pb and 0.24-2.00 μg L^{-1} Cu) [22,25,32], our LoD for Cd in sample solution was similar, while for Cu, Pb and Zn poorer. The difference is explained by the fact that our LoDs were calculated from the parameters of the standard addition curve, while the

literature values refer to synthetic solutions. In comparison with other widely used spectrometric techniques (ICP-OES, GFAAS), our LoDs were comparable for Cd and Pb (0.2-1 μg L^{-1} (Cd), 1-5 μg L^{-1} (Pb) [33]) and poorer for Zn and Cu (0.09-1 μg L^{-1} (Zn), 0.2-2 μg L^{-1} (Cu) [33]). The optimized DPASV method makes possible determination of Cd and Pb as toxic elements in mushrooms/food supplements, since LOQ found by us provide quantification starting from 0.04 μg g^{-1} Cd and 0.5 μg g^{-1} Pb, which are much lower than the maximum allowed concentration in mushroom supplements (1 μg g^{-1} Cd and 3 μg g^{-1} Pb) [29]. The use of DPASV method, on the other hand, provides the advantage of simultaneous multi-element determination compared to AAS and low analysis costs.

Accuracy

Method accuracy was assessed through a spike recovery test (n=3 spikes). A good agreement between found and theoretical amounts was obtained for 95% confidence level. Mean recoveries were (%): 104±6 Cu, 102±4 Cd, 100±7 Pb and 102±5 Cu and included in all cases the 100% value.

Analysis of mushroom samples

Results obtained for metal concentration in mushroom food supplements and mushroom expressed in dry weight are summarized in Table 3.

Table 3. Metal concentrations in the analyzed mushroom and mushroom supplements

Mushrooms	Mean±C.I.[a] (μg g^{-1} dry weight)			
	Zn	Cd	Pb	Cu
Food supplements				
Reishi	32.7±1.2	0.06±0.01	2.63±0.34	1.44±0.12
Chaga	49.0±2.0	0.40±0.06	3.60±0.12	4.00±0.72
Shiitake	10.4±0.5	0.57±0.16	2.34±0.18	6.62±4.90
Mixture	46.4±7.2	0.09±0.01	3.27±0.16	21.5±5.8
Fresh mushroom				
King bolete	102±4	0.69±0.02	1.83±0.05	24.1±1.4
RSD[b] (%)	1.5-7.4	1.4-11.6	1.1-5.2	2.4-10.9

[a] C.I. is the confidence interval for 95% confidence level (n=3 successive measurements).
[b] RSD (%) is the relative standard deviation.

RSD for all four metals varied between 1.1 and 11.6 %, thus proving precision better than 10% for Zn and Pb and up to 11% for Cd and Cu, fulfilling the recommendation of AOAC in terms of precision [34].

The most abundant metal was found to be Zn (10.4-102 μg g^{-1}), in agreement with the characteristic of mushrooms to accumulate Zn [2,35-

37], while Cu was found in lower concentrations (1.44-24.1 μg g^{-1}). The high concentration of Zn is beneficial as human organism has greater need for Zn than Cu [29]. Among the analyzed samples, the highest amounts of both Zn and Cu were found in King bolete mushroom (102 μg g^{-1} Zn and 24.1 μg g^{-1} Cu), followed by the mixture of medicinal mushroom capsules (46.4 μg g^{-1} Zn and 21.5 μg g^{-1} Cu).

Cadmium in the edible mushroom (0.69 μg g^{-1}) was found to be only slightly higher than in supplements (0.06-0.57 μg g^{-1}).

Lead was in the range 1.83-3.60 μg g^{-1}. Thus, the benefit of high concentration of essential elements is limited to some extent.

The comparison of results obtained for the edible King bolete with dietary supplements revealed no evident discrepancy in terms of Cd, Pb and Cu, unlike Zn, for which the content was approximately twofold higher, namely 102 μg g^{-1} for the King bolete, versus 10.4-49.0 μg g^{-1} in dietary supplements. Much higher concentrations of Zn, Cd and Pb were reported in other studies for the same mushroom species (55.5-283.9 μg g^{-1} Zn, 0.66-283.9 μg g^{-1} Cd and 0.14-86 μg g^{-1} Pb), while similar concentrations were found for Cu (13.7-55.7 μg g^{-1}) [1-2,10,18,37].

Assessment of Cu, Pb, Zn and Cd intake via mushroom and mushroom supplements consumption

The maximum allowed concentrations of Cd and Pb in mushroom supplements are 1.0 μg g^{-1} and 3.0 μg g^{-1} dry weight according to Commission Regulation (EC) No 629/2008 [29], and 0.2 μg g^{-1} and 0.3 μg g^{-1} wet weight respectively in cultivated fungi set in Commission Regulation (EC) No 1881/2006 [28]. Assuming 90 % moisture content in fresh mushrooms, the maximum acceptable levels expressed in dry mass become 2 μg g^{-1} Cd and 3 μg g^{-1} Pb in King bolete mushroom. As show data in Table 3, Cd concentrations in all samples were below the set values, while Pb limit was exceeded in the case of Chaga and Mixture supplement by up to 20%.

Table 4 presents the intake of Zn and Cu as essential elements, and Cd and Pb as toxic heavy metals from mushroom supplements *via* the maximum dose recommended by the manufacturer (5.4 g day^{-1} Reishi, 2 g day^{-1} Chaga, 4 g day^{-1} Shiitake and 2.46 g day^{-1} mushroom Mixture) and a serving of 100 g mushroom per day, respectively. Values for Cd were compared to the provisional tolerable monthly intake (PTMI) of 0.025 mg Cd/kg body weight (1.5 mg for 60 kg body weight) set by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [30]. According to data in Table 4 the limit was not exceeded in none of the cases.

For the evaluation of Pb intoxication *via* contaminated food, JECFA advises a dose-response analysis, separately for adults and children (1-4

years old) [30]. Accordingly, for a 60 kg adult the exposure to 0.02 μg Pb/kg body weight per day (1.2 μg/day) represents a negligible health risks, 1.2 μg Pb/kg body weight per day (72 μg/day) is associated with an increase of systolic blood pressure and 3 μg Pb/kg body weight per day (180 μg/day) is proven to cause systolic blood pressure increase associated with increase in the risks of ischemic heart disease and cerebrovascular stroke. The comparison of these values with Pb concentrations found in our samples emphasized a non-existing/low health risk (5.36-18.3 μg/day) even for samples in which Pb slightly exceeded the maximum acceptable levels.

Table 4. Metals intake[a] and recommended daily allowances

Mushrooms	Zn		Cu		Cd	Pb
	mg day^{-1}	RDA[b] (%)	mg day^{-1}	RDA (%)	mg $month^{-1}$ [c]	μg day^{-1}
Food supplements						
Reishi	0.18	1.8	0.01	1	0.01	14.2
Chaga	0.10	1.0	0.01	1	0.02	7.20
Shiitake	0.04	0.4	0.03	3	0.07	5.36
Mixture	0.11	1.1	0.05	5	0.01	8.04
Fresh mushroom						
King bolete	1.02	10.2	0.24	24	0.21	18.3

[a] Consumption of 100 g fresh King bolete (90% moisture)/day, 5.4 g/day Reishi, 2 g/day Chaga, 4 g/day Shiitake and 2.46 g/day Mixture.
[b] RDA is the Recommended Daily Allowance (10 mg/day Zn and 1 mg/day Cu [15]).
[c] Calculated for a 60 kg adult and month consisting of 30 days.

Zn intake through supplements expressed as Recommended Daily Allowance (%RDA) was in the range 0.4 (Shiitake)-1.8 (Reishi) and much higher in the case of the King bolete (10.2%). The situation was very similar in the case of Cu, with higher %RDA for the fresh mushrooms (24%) than in supplements (1% for Reishi and Chaga - 5% for the Mixture).

CONCLUSIONS

Anodic stripping voltammetry with hanging mercury drop electrode was optimized for the determination of Zn, Cd, Pb and Cu in mushroom food supplements and fresh mushroom in terms of multi-element standard solution used in the standard addition method, Hg drop size and deposition time. Performances of the methods were assessed regarding LoD, LoQ, accuracy and precision and were found to be satisfactory for the analyses of mushroom samples after mineralization.

The comparison of metal concentration in food supplements and the edible King bolete fungus revealed no difference in terms of Cd, Pb and Cu content, while Zn was more abundant in the fresh mushroom. It has been found that King bolete represents a significant source of Cu and Zn compared to the recommended daily allowance, while Cd and Pb pose no health risk for both food supplements and fresh mushroom.

EXPERIMENTAL SECTION

Reagents, stock solutions and samples

Nitric acid 69%, perchloric acid 70%, hydrochloric acid fuming 37% and stock solutions of Zn, Cd, Pb and Cu (1000 mg L^{-1}) were purchased from Merck (Darmstadt, Germany); acetic acid ≥99.5% and sodium hydroxide ≥99% were purchased from Sigma-Aldrich (Hamburg, Germany). All dilutions throughout this study were made using doubly distilled water obtained with Fistreem Cyclon Double (Bi-) Distiller (United Kingdom).

The analyzed samples were mushroom supplements: Reishi (*Ganoderma lucidum*) as tablets, Chaga (*Inonotus obliquus*), Shiitake (*Lentinula edodes*) as dried powders and a Mixture of medicinal mushroom extracts as capsules recommended for their benefit associated to overall good health, balance in the organism, memory, respiratory function and resistance to the aging process, as well as a fresh mushroom, King bolete (*Boletus edulis*), from local market.

Instrumentation

Voltammetric measurements were carried out using the 797 VA Computrace, Metrohm AG (Switzerland) instrument. The voltammetric analyzer was controlled by computer, using the VA Computrace software Metrohm. Operating conditions are given in Table 5.

Table 5. Working conditions for DPASV measurements

Electrode	HMDE - Hanging mercury drop electrode
Drop size	4-8, optimal 6
Stirring rate	2000 rpm
Initial Ar purging	180 s
Deposition potential	-1.2 V
Deposition time	60-360 s, optimal 60 s
Equilibration time	5 s
Start potential	-1.2 V
End potential	0.08 V
Pulse amplitude	0.05 V
Pulse time	0.04 s
Voltage step	0.005 V
Voltage step time	0.3 s
Scan rate	0.0168 V s^{-1}
Quantification	Peak height
Oxidation potentials (vs. Ag/AgCl)	
Zn	(-0.90)-(-1.00) V
Cd	(-0.56)-(-0.58) V
Pb	(-0.38)-(-0.41) V
Cu	(-0.03)-(+0.01) V
Supporting electrolyte	1 mL acetate buffer (pH 4.6, ionic strength 0.1) and 9 mL H_2O

Sample preparation

All samples were dried to constant weight, ground in a mortar (if necessary) and sieved to ≤ 63 μm. The King bolete analytical sample was constituted of whole mushroom (stem and cap). An amount of 1 g sample was digested with 20 mL HNO_3 and 5 mL $HClO_4$, by heating on a sand bath. The digest was filtered and diluted to 50 mL in volumetric flasks.

Sample measurement

Quantitative analyses were realized using 0.5 mL aliquots from the digested sample added to 10 mL supporting solution (9 mL H_2O and 1 mL acetate buffer). Steps of DPASV: deaeration of electrolyte by Ar purging for 180 s; analyte deposition at HMDE at -1.2 V under stirring, equilibration for 5s; anodic stripping and voltammogram recording in the range (-1.2) - (0.08) V. Simultaneous determination of Zn, Cd, Pb and Cu was performed using the standard addition method (n=4 spikes) under the optimized conditions given in Table 5.

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